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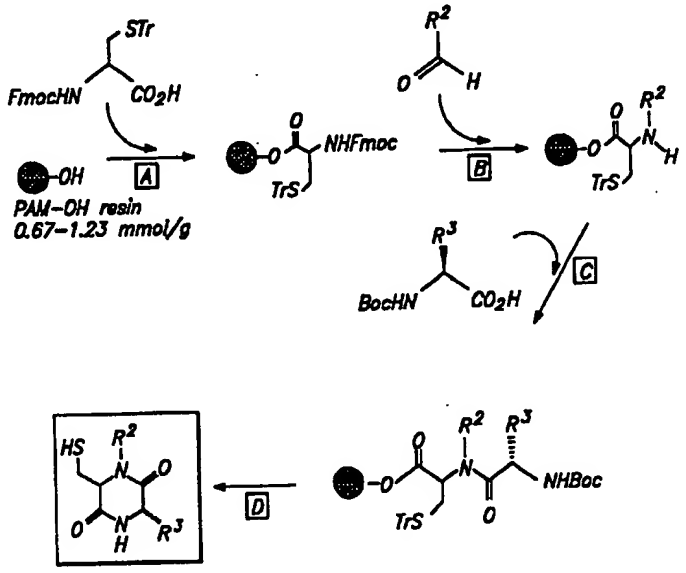
(54) Title: METHODS FOR THE SYNTHESIS OF DIKETOPIPERAZINES

(57) Abstract

Libraries of diverse diketopiperazines bound to a support and methods for synthesizing diketopiperazines on a solid support are described. These libraries have utility in the area of drug design as they can be screened against biological substances to identify compounds which have desirable biological activity.



37

A. FmocAA, DIC, DMAP, DMF, 5h

B. 30% piperidine
–aldehyde, $NaCNBH_3$, $HC(OMe)_3$,
1% HOAc or MeOH

C. BocAA, HATU, DIEA, DCM/DMF, 2x12h

D. 95% TFA/TES
1% HOAc IN toluene 18h

Figure 4 is a schematic illustrati n of the preparation of a combinatorial library of soluble N-alkylated diketopiperazines and the components used in each step.

Figure 5 is a schematic illustration of the preparation of a combinatorial library of soluble N-alkylated diketopiperazines and the components used in each step.

Figure 6 illustrates the formation of diketopiperazines using cysteine as the first amino acid.

Figures 7-9 illustrate methods for forming polycyclic structures based on diketopiperazines.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The description of the invention is provided as indicated by the following outline. In addition, Section I provides for a glossary of terms to facilitate the description of the invention. A number of terms and abbreviations are defined to have the general meanings indicated as used herein to describe the invention.

OUTLINE

I. Terminology

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

"Activation" or "activating agent" refers to a reagent or energy source which selectively converts a functionality (typically, a carboxyl group) to an activated functionality (typically, an activated ester) which is capable of coupling to a second functionality. For example, carboxyl group can be activated through various means including, but not limited to, the production of the corresponding -OPfp ester through treatment with DCC and pentafluorophenol (*see, e.g.*, Kisfaludy and Schon (1983) Synthesis 325-327) or the trifluoroacetate salt of pentafluorophenoxide and pyridine (*see* Green and Berman (1990) Tetrahedron Lett. 31:5851-5852). Another preferred form of activated carbonyl is the N-carboxyanhydride group, which can be produced via methods well known in the art. The activated carboxyl group can then be coupled, for example, to an amino group to produce an amide linkage.

"Activated ester" refers to an ester capable of reacting with an amine group to produce an amide linkage. Typically, the carbonyl carbon of an activated ester possesses a higher degree of positive charge character than the carbonyl carbon of an unactivated ester, i.e., a lower alkyl ester.

"Acyl" denotes groups -C(O)R, where R is alkyl or substituted alkyl, aryl, or substituted aryl as defined below.

"Alkoxyl" denotes the group -OR, where R is lower alkyl, substituted lower alkyl, aryl, substituted aryl, aralkyl or substituted aralkyl as defined below.

"Alkylthio" denotes the group -SR, where R is lower alkyl, substituted lower alkyl, aryl, substituted aryl aralkyl or substituted aralkyl as defined below.

"Amido" denotes the group -C(O)NRR', where R and R' may independently be hydrogen, lower alkyl, substituted lower alkyl, aryl, substituted aryl as defined below or acyl.

"Amino" denotes the group NRR', where R and R' may independently be hydrogen, lower alkyl, substituted lower alkyl, aryl, substituted aryl as defined below or acyl.

"Amino Acid Derivative" refers to an amino acid, preferably an α- or β-amino acid, which has been modified by the addition of one or more protecting groups, such as 9-fluorenylmethyloxycarbonyl (Fmoc), benzyl or t-butoxycarbonyl (BOC), and/or activating groups or by its coupling to a solid support.

"Aralkyl" refers to the group -R-Ar where Ar is an aryl group and R is straight-chain or branched-chain aliphatic group. Typical arylalkyl group will comprise from 7 to 20 carbon atoms. Aralkyl groups can optionally be unsubstituted or substituted with, *e.g.*, halogen, lower alkyl, lower alkoxy, lower alkylthio, trifluoromethyl, lower acyloxy, and hydroxy. Preferred aralkyl groups include benzyl, hydroxybenzyl, methylbenzyl, chlorobenzyl, bromobenzyl, iodobenzyl, thiobenzyl, aminobenzyl, napthylmethyl and hydroxynapthylmethyl.

"Aryl" or "Ar" refers to an aromatic carbocyclic group having at least one aromatic ring (*e.g.*, phenyl or biphenyl) or multiple condensed rings in which at least one ring is aromatic, (*e.g.*, 1,2,3,4-tetrahydronaphthyl, naphthyl, anthryl, or phenanthryl). Typical aryl groups will comprise from 6 to 14 carbon atoms and preferably from 6 to 10 carbon atoms. Particularly preferred substituents are phenyl and napthyl.

"Substituted aryl" refers to aryl optionally substituted with one or more, typically 1 to 3, functional groups, *e.g.*, halogen, lower alkyl, lower alkoxyl, lower alkylthio, trifluoromethyl, lower acyloxyl, hydroxyl and the like. Preferred groups include methylphenyl, chlorophenyl, iodophenyl, bromophenyl, 4-hydroxyphenyl, thiophenyl, 4-chlorothiophenyl, 2-methylthiophenyl and 4-methylsulfonylphenyl.

"Aryloxyl" denotes groups -OAr, where Ar is an aryl or substituted aryl group as defined below.

"Dipeptide Derivative" refers to a dipeptide which has been modified by the addition of one or more protecting groups, such as 9-fluorenylmethyloxycarbonyl (Fmoc), benzyl or t-butoxycarbonyl (BOC), and/or activating groups or by its coupling to a solid support.

"Exogenous base" refers to nonnucleophilic bases such as alkali metal acetates, alkali metal carbonates, alkaline metal carbonates, alkali metal bicarbonates, tri(lower alkyl) amines, and the like, for example, sodium acetate, potassium bicarbonate, calcium carbonate, diisopropylethylamine, triethylamine, and the like.

"Halogen" refers to bromine, chlorine, and/or iodine atoms.

"Heteroaryl" or "HetAr" refers to an aromatic carbocyclic group having a single ring (*e.g.*, pyridyl or furyl) or multiple condensed rings (*e.g.*, naphthyridinyl, quinoxalyl, quinolinyl, indolizinyl or benzo[b]thienyl) and having at least one hetero atom, such as N, O or S, within the ring. Typically, such heteroaryl groups will comprise from 4 to 20 carbon atoms and from 1 to 3 hetero atoms.

"Substituted heteroaryl refers to heteroaryl substituted with one or more, typically, 1 to 3, *e.g.*, halogen, lower alkyl, lower alkoxy, lower alkylthio, trifluoromethyl, lower acyloxy, and hydroxy. Preferred heteroaryl groups include indolyl, methylindolyl, imidazolyl, N-methyl-imidazolyl and methylimidazolyl.

"Heteroarylalkyl" refers to the group -R-HetAr where HetAr is an heteroaryl group and R is straight-chain or branched-chain aliphatic group, typically of from 1 to 10 carbon atoms. Heteroarylalkyl groups can optionally be unsubstituted or substituted with, *e.g.*, halogen, lower alkyl, lower alkoxyl, lower alkylthio, trifluoromethyl, lower acyloxy, and hydroxy. Preferred heteroaralkyl groups include 3-indolylmethyl and 2-imidazolylmethyl.

"Hydroxyl" refers to the group -OH.

"Cleavable linking arms" refer to linking arms wherein at least one of the covalent bonds of the linking arm which attaches the compound comprising the diketopiperazine group to the solid support can be readily broken by specific chemical reactions thereby providing for compounds comprising diketopiperazine groups free of the solid support ("soluble compounds"). The chemical reactions employed to break the covalent bond of the linking arm are selected so as to be specific for bond breakage thereby preventing unintended reactions occurring elsewhere on the compound. The cleavable linking arm is selected relative to the synthesis of the compounds to be formed on the solid support so as to prevent premature cleavage of this compound from the solid support as well as not to interfere with any of the procedures employed during compound synthesis on the support.

Suitable cleavable linking arms are well known in the art and Figures 1A-1D illustrates several embodiments of such linking arms. Figure 1A illustrates a cleavable Sasrin resin comprising polystyrene beads and a cleavable linking arm as depicted therein which linking arm is cleaved by strong acidic conditions such as trifluoroacetic acid. Cleavage results in

breakage at the vertical line interposed between the oxygen and carbonyl moieties of the ester so as to provide for a compound terminating in a carboxylic acid.

Figures 1B and 1C illustrate cleavable TentaGel AC and TentaGel PHB resins respectively, each comprising a polystyrene bead and the cleavable linking arm depicted therein both of which are cleaved by strong acidic conditions such as trifluoroacetic acid. Cleavage results in breakage at the vertical line interposed between the oxygen and carbonyl moieties of the ester so as to provide for a compound terminating in a carboxylic acid.

Figure 1D illustrates a cleavable TentaGel RAM resin comprising a polystyrene bead and a cleavable linking arm depicted therein which is cleaved by strong acidic conditions such as trifluoroacetic acid. Cleavage results in breakage at the wavy line interposed between the nitrogen and the benzhydryl carbon of the linking arm so as to provide for a compound terminating in an amide group. In this case, this linking arm facilitates formation of the amide bond by stabilizing the intermediate carbonium ion on the carbon atom between the two aromatic groups. Such stabilization permits selective bond cleavage as compared to bond cleavage for other amide groups of the compound comprising a pyrrolidinyl group.

Reversible covalent cleavable linkages can be used to attach the molecules to the support. Examples of suitable reversible chemical linkages include (1) a sulfoester linkage provided by, e.g., a thiolated tagged-molecule and a N-hydroxy-succinimidyl support, which linkage can be controlled by adjustment of the ammonium hydroxide concentration; (2) a benzylhydryl or benzylamide linkage provided by, e.g., a Knorr linker, which linkage can be controlled by adjustment of acid concentration; (3) a disulfide linkage provided by, e.g., a thiolated tagged-molecule and a 2-pyridyl disulfide support (e.g., thiolsepharose from Sigma), which linkage can be controlled by adjustment of the DTT (dithiothreitol) concentration; and (4) linkers which can be cleaved with a transition metal (e.g. HYCRAM).

The linker may be attached between the tag and/or the molecule and the support via a non-reversible covalent cleavable linkage. For example, linkers which can be cleaved photolytically can be used. Preferred photocleavable linkers of the invention include those recited in U.S. Patent Application Serial No. 08/374,492, filed, January 17, 1995, 6-nitro-veratry-oxycarbonyl (NVOC) and other NVOC related linker compounds (see PCT patent publication Nos. WO 90/15070 and WO 92/10092; see also U.S. patent application Serial No. 07/971,181, filed 2 Nov. 1992, incorporated herein by reference); the ortho-nitrobenzyl-based linker described by Rich (*see* Rich and Gurwara (1975) J. Am. Chem. Soc. 97:1575-1579; and Barany and Albericio (1985) J. Am. Chem. Soc. 107: 4936-4942) and the phenacyl based linker discussed by Wang. (*see* Wang (1976) J. Org. Chem. 41:3258; and Bellof and Mutter (1985) Chimia 39:10).

"Lower alkyl" refers to a cyclic, branched or straight chain, alkyl group of one to eight carbon atoms. This term is further exemplified by such groups as methyl, ethyl, n-propyl, i-propyl, *n*-butyl, *t*-butyl, *i*-butyl (or 2-methylpropyl), cyclopropylmethyl, *i*-amyl, *n*-amyl, and hexyl. Preferred groups are methyl, *sec*-butyl, *iso*-butyl and *iso*-propyl.

"Substituted lower alkyl" refers to lower alkyl as just described including one or more (typically 1 to 3) functional groups such as lower alkyl, aryl, aralkyl, acyl, halogen, hydroxyl, amino, mercapto and the like. These groups may be attached to any carbon atom of the lower alkyl moiety. Preferred groups are 2-guanidinopropyl, 2-carboxymethyl, 2-amidomethyl, thiomethyl, 2-carboxyethyl, 2-amidoethyl, 3-imidazolyl-methyl, 4-aminobutyl, 3-hydroxyl-4-aminobutyl, 2-(methylthio)ethyl, hydroxymethyl and 1-hydroxyethyl.

"Non-cleavable linking arms" refer to linking arms wherein the covalent bonds linking the compound comprising a diketopiperazine to the solid support can only be cleaved under conditions which chemically alters unintended parts of the structure of the compound attached thereto.

"Stereoisomer" refers to a chemical compound having the same molecular weight, chemical composition, and constitution as another, but with the atoms grouped differently. That is, certain identical chemical moieties are at different orientations in space and, therefore, when pure, have the ability to rotate the plane of polarized light. However, some pure stereoisomers may have an optical rotation that is so slight that it is undetectable with present instrumentation. The compounds of the present invention may have one or more asymmetrical carbon atoms and therefore include various stereoisomers. All stereoisomers are included within the scope of the invention.

"Substantially Homogeneous" refers to collections of molecules wherein at least about 80%, preferably about 90% and more preferably about 95%, of the molecules are a single compound or stereoisomer therof.

"Substrate" or "support" refers to a material or group of materials having rigid or semi-rigid structures. These materials may take the form of beads, gels, resins, pins, microspheres, rings, of flat surfaces. The substrate or support surface may further be divisible into two or more regions upon which chemically diverse structures may be bound. Other forms will be known to those of skill in the art.

"Thiol" or "mercapto" refers to the group -SH.

Abbreviations

The following abbreviations will be used herein. It will be recognized that these abbreviations are of common usage in the chemical arts.

BOC:	t-Butoxycarbonyl.
BOP:	Benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate.
PyBOP:	Benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate.
DCC:	Dicyclohexylcarbodiimide.
Fmoc:	Fluorenylmethyloxycarbonyl.
TFA:	Trifluoroacetic acid.
DMF:	Dimethylformamide.

DIEA: Diisopropylethylamine.
TEA: Triethylamine.
DBU: 1,8-Diazabicyclo[5.4.0]undec-7-ene.
DMAP: N,N-Dimethylaminopyridine.
DIC: Diisopropylcarbodiimide.
HOBT: 1-Hydroxybenzotriazole.
HATU: [O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium]hexafluorophosphate.
HBTU: 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uroniumhexafluorophosphate.
Trt: Triphenylmethyl or trityl.
DMSO: Dimethylsulfoxide.
NMP: N-Methylpyrollidine.

II. Overview

In one embodiment, the present invention includes methods for synthesizing N-linked and C-linked diketopiperazines on a solid support. In addition, a method for preparing soluble N-alkylated diketopiperazines is described. For each of these methods, a first amino acid derivative is attached to a solid support to form a bound first amino acid derivative. The bound first amino acid derivative can be reacted with a second amino acid derivative under conditions effective to form a peptide bond, so that a bound dipeptide derivative is formed. This bound dipeptide derivative is then reacted under conditions effective to cyclize the bound derivative to form thereby a bound N-linked diketopiperazine. Alternatively, when the first amino acid derivative comprises an N-protected carboxylalkyl amino acid, the methods described herein can be utilized to prepare C-linked diketopiperazines. If the first amino acid derivative is instead reductively aminated, soluble N-alkylated diketopiperazines can be produced via cyclization of the dipeptide derivative and concomitant cleavage from the resin. Components common to each of these methods are described below, followed by more detailed descriptions of the specific embodiments.

A. The Amino Acids and Derivatives Thereof

The methods of the present invention produce diketopiperazines, and derivatives thereof, typically from the coupling of two amino acids. The amino acids and derivatives thereof used in the present invention include the twenty naturally occurring α-amino acids, in either their D- or L-enantiomeric forms. Unnatural amino acids such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids are also suitable components for the diketopiperazines of the present invention. Examples of unnatural amino acids include: 4-hydroxyproline, O-phosphoserine, 3-methylhistidine, 5-hydroxylysine, and other similar amino acids and imino acids (*e.g.*, 4-hydroxyproline). Thus, the present invention includes methods for synthesizing diketopiperazines such as *cyclo*-L-Ala-L-Lys in addition to its unnatural derivatives, such as, *cyclo*-L-Ala-L-(5-hydroxyLys). Thus, the present invention specifically includes the use of all α-amino acid derivatives in addition to the derivatives of the twenty naturally occurring amino acids just described. Techniques for making α-amino acids are well known in the chemical arts and are described in such common references as, *e.g.*, those by Williams.[13]

The side chain (for example, designated as R^1 in amino acid derivative **2** or R^2 in amino acid derivative **4**, see Reaction Scheme I) of the amino acid may include hydrogen, alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, acyl, alkoxyl, aryloxyl, mercapto, alkylthio, arylthio, hydroxyl, cyano, halogen, amino, and amido. Preferred substituents are any of those found on naturally occurring amino acids, such as benzyl, hydroxymethyl, thiomethyl, methyl, hydrogen, *iso*-propyl, *iso*-butyl, imidazolylmethyl, indolylmethyl, 4-aminobutyl, ethoxyl, 2-methylthioethyl, 3-guanidylpropyl, 2-carboxyethyl, 2-amidoethyl, or the like. Other amino acid derivatives include α,α-disubstituted amino acids.

According to some embodiments, the side chain of at least one of the amino acid derivatives will comprise a chelation group. A chelation group

is a chemical functionality or a combination of chemical functionalities which are capable of forming coordination complexes with metal ions, for example, Cu^{+2}, Zn^{+2}, Co^{+2}, or Ni^{+2}. Preferred chelation groups include, but are not limited to, carboxylates, malonates, hydroxamates, and thiolates, such as mercaptoketones, and mercaptoalcohols. In some embodiments, for example with malonate or carboxylate, the chelation group, typically in a protected format, will be present throughout the synthetic sequence. According to other embodiments, for example with hydroxamates, the chelation group will be introduced via a post-cyclization transformation as described in more detail below.

The amino acid derivatives described herein may include one or more protecting groups to prevent unwanted side reactions during various steps of the synthesis of the desired diketopiperazines. Such protecting groups and methods for attaching and removing these groups are known commonly in the art, *see, e.g.*, Green and Wuts,[14] and Grant[15]. Preferred protecting groups include Fmoc and BOC groups for protecting the α-amino group of the second amino acid derivative from unwanted side reactions. Protecting groups may also include photolabile or photoreactive protecting groups, such as those described in co-pending U.S. Patent Applications Serial Nos. 07/624,120, 08/374,492,, and U.S. Patent No. 5,143,854 to Pirrung, *et al.*, each of which incorporated herein by reference.

B. The Solid Support

The support upon which the diketopiperazines are synthesized may be any solid support which is compatible with peptide synthesis, such as those described in Grant and Atherton.[16] Generally, these supports may comprise glass, latex, cross-linked polystyrene and other similar polymers and resins, gold and other colloidal metal particles. Other materials will be familiar to those of skill in the art. A preferred support includes polymer-supported anisaldehyde resins, such as resin-bound 2-methoxy-4-oxy-

anisaldehyde or 4-oxyanisaldehyde, which can be made from commercially available resin backbones such as TentaGel S AC or TentaGel PHB (Rappe Polymere, Tübingen, Germany) by oxidation using conventional methods such as reaction of the resin backbone with pyridinium sulfur trioxide or dimethylsufoxide/oxalyl chloride. Another preferred support is a polymer-supported bromoacetamide resin, which can be prepared from commercially available backbone resins TentaGel S NH_2 or RAM resin (Rappe Polymere, Tübingen, Germany) or Pharmacia Mono A resin (Pharmacia, Piscataway, N.J.) by reaction with bromoacetic acid using standard methods. Yet another preferred support includes a resin-bound Knorr-type linker, *i.e.*, a benzhydryl or benzylamine derivative which releases an amide or acid upon cleavage. This may be attached to the resin by the reaction of the resin with *p*-[(R,S)-a-[1-(9H-fluoren-9-yl)-methyloxyformamido]-2,4-dimethoxy-benzyl]phenoxyacetic acid, benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate and diisopropyl-ethylamine in DMF.

The surface of the support may also comprise protecting groups such as those just described above to block one or more regions from becoming derivatized during one or more steps in the synthesis of diketopiperazines, as discussed in greater detail below. For example, where the surface of the support is derivatized with amine groups, amine protecting groups such as Fmoc may be employed to prevent reactions in those areas of the support surface so protected. The support surface may also include one or more areas protected by the photolabile groups, such as those described in Pirrung, *supra*, and in co-pending U.S. Patent Applications Serial Nos. 08/310,510, filed September 22, 1994 and 07/971,181 filed November 2, 1991. The use of photolabile protecting groups allows the employment of photolithographic techniques to produce a support having a large density of diverse diketopiperazine compounds at known locations on the support surface.

As noted above, the support may contain linker or spacer molecules which anchor the first amino acid derivatives to the support surface. A

variety of linkers are well known in the art,[17] and are described in Applicants' co-pending U.S. Patent Application Serial No. 08/146,886, which is incorporated herein by reference. Generally the linkers are chosen to have lengths which allow the molecules to which they are attached sufficient exposure to reagents and/or receptors which may be under study. The linkers may also be chosen to impart desired hydrophobic, hydrophilic, or steric properties. For example, linkers with bulky side chains, such as *tert*-butyl side chains, may be used to provide rigidity or control spacing on the support. The linker will typically include a functional group to which the first amino acid derivative is attached. This functional group may be protected initially so as to permit activation of the surface-bound linkers in selected areas of the support only. Preferred linkers include anisaldehyde derivatives such as 2- or 4-alkoxy- benzylamine or benzyl alcohol, bromoacetic acid and the Knorr linker or a similar linker, *i.e.*, a benzhydryl or benzylamine derivative which releases an amide or acid upon cleavage. Also preferred are oligonucleotide linkers comprising one or more restriction sites which may be cleaved selectively by a restriction enzyme.

C. Coupling Conditions

For each of the methods described herein, a second amino acid derivative is coupled to a bound first amino acid derivative (optionally, mono-alkylated as in the preparation of N-alkylated diketopiperazines) to yield a bound dipeptide derivative. Prior to coupling the second amino acid to the bound first amino acid, the protecting group, if present, on the amino group of the bound first amino acid should be removed. Typically, standard deprotection conditions known in the art can be used. For example, removal of an Fmoc may be performed with 20% to 55% of a secondary amine base such as piperidine in a polar, aprotic solvent such as DMF, methylene chloride or N-methylpyrrolidine. Typically, deprotection is achieved in about 5 minutes to one hour, but this time may be varied if oligonucleotide tagged libraries are used (see below) which may be sensitive

to these reaction conditions. Depending on the choice of amino acid, solvent and base, a precipitate comprising an amine salt may occur after standing for a short period. In a preferred embodiment, the Fmoc protecting group of the first amino acid is removed by reaction of the bound derivative with a 30% piperidine/dimethylformamide solution.

A tertiary amine base, such as DBU, may also be used to remove the Fmoc group. Typically a solution of about 2% to 10%, preferably 5% DBU, in a polar, aprotic solvent such as DMF is used. However, if oligonucleotide tags are used, care should be taken as DBU has been noted to cause base modification.[18] Also, following removal of Fmoc with DBU, the resin should be washed immediately to remove reactive Fmoc intermediates. Typically, these reactions are performed at room temperature, although the reaction mixture may be heated or cooled to enhance or retard the rate of reaction.

The bound first amino acid derivative having a free (i.e., unprotected) terminal amino group is next reacted with a second amino acid derivative under conditions effective for the formation of the corresponding dipeptide. Generally, the second amino acid derivative will include a protecting group for the α-amino moiety of the amino acid. A preferred protecting group is Fmoc or BOC. A photolabile protecting group such as described above may also be employed. Many other protecting groups for the α-amino group are known in the art (*see, e.g.*, Green and Wuts, Grant or Atherton, *supra*).

According to some embodiments, the dipeptide is prepared through the coupling of an activated amino acid to the bound amino acid. For example, the carboxyl group of the second amino acid can be activated by conversion to an activated ester, such as the corresponding -ODhbt, -OSu or -OPfp ester or to an amino acid fluoride. Often these activated carboxyl groups are formed *in situ*. Techniques for producing these activated esters are well known in the art.

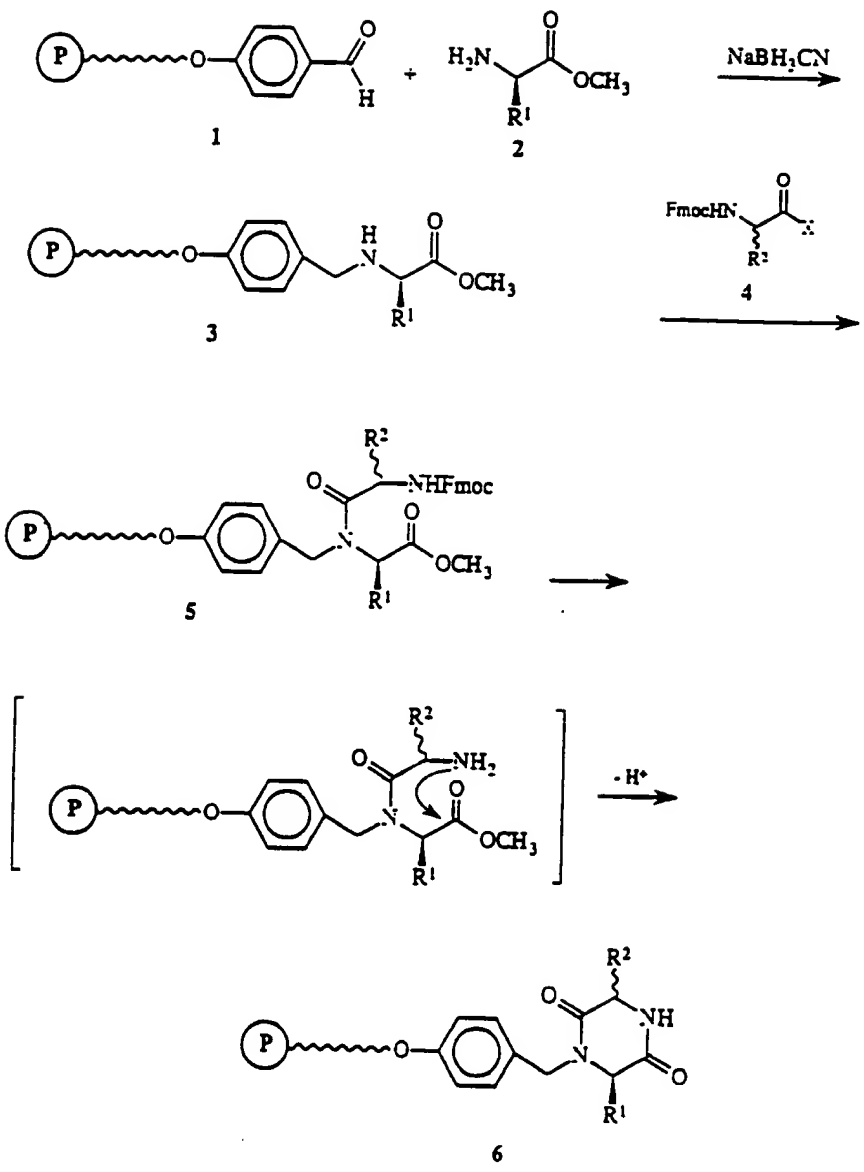
Other embodiments will utilize a suitable coupling catalyst, such as DCC, DIC, HOBT, HATU, HBTU, BOP and PyBOP, to effect formation of the dipeptide. Typically an excess of coupling reagent is used, with quantities ranging from 2 equivalents to 10 equivalents or more. Preferably 3 to 8 equivalents are employed. Often the degree of excess is determined with respect to the reactivity of the chemical species being coupled. Polar, aprotic solvents such as DMF, NMP, DMSO and methylene chloride are preferred. Reaction times may vary from 0.5 to 3 hours to overnight, and temperatures may vary from room temperature to reflux. In a preferred embodiment, the coupling is effected through the use of BOP, and optionally an exogenous base, such as DIEA.

D. Cleavage Conditions

N-Linked and C-linked diketopiperazines can be removed from the support by cleavage from the linker. Again, the conditions effective to cause removal from the resin or linker will depend on the type of resin or linker chosen. Generally the desired diketopiperazine is removed by acid hydrolysis, using a strong acid such a TFA in water. Typical TFA/H_2O ratios are between about 80/20 to 99/1, with 95/5 preferred.

III. Synthesis of N-Linked Diketopiperazines

According to one embodiment of the present invention, N-linked diketopiperazines are prepared through the methods exemplified in Reaction Scheme I below:



Reaction Scheme I

A. Formation of the Bound First Amino Acid Derivative

The synthesis of N-linked diketopiperazines on a solid support using a method of the invention is shown in Reaction Scheme I. An anisaldehyde linker 1 is bound to a polymer support P, *e.g.*, by reaction of 4-hydroxybenzaldehyde with a chloromethyl resin. The linker is coupled to

the N^{α} group of a first amino acid derivative **2** to form an imine, which is subsequently reduced to the corresponding amine **3** using a reducing agent, such as sodium borohydride or sodium cyanoborohydride. Typically, the carboxyl group of the first amino acid derivative is protected with a group which is stable to the reducing agent employed, so as to prevent unwanted reduction of the carboxyl moiety. For example, as shown in Reaction Scheme I, the carboxyl group is protected as the methyl ester. Other combinations of protecting groups and reducing agents will be apparent to those of skill in the art (*see*, Green and Wuts, *supra*).

Generally formation of the imine is performed in a solvent which is capable of solvating the reactive species involved, such as a polar solvent, under conditions effective to remove water as this is generated by the formation of the imine. For example, solvents having a higher boiling point than water may be used in conjunction with a trap so that on reflux of the solvent, the water is removed from the system by collection in the trap. Alternatively, drying agents, such as molecular sieves, may be used to trap water *in situ* upon its formation. Preferred dehydrating agents include molecular sieves, magnesium sulfate, sodium sulfate, trimethyl orthoformate, zinc chloride, and the like. More preferably, the dehydrating agent is in a form which can be easily washed away from the solid support or is even used as the solvent. Most preferably, the dehydrating agent comprises trimethylorthoformate.

Typically, an excess of both the amine and the reducing agent is used. Preferably, at least 5 equivalents of amine and/or at least 5 equivalents of reducing agent is used. More preferably, the alkylation is performed with about 10 equivalents or more of amine and about 10 equivalents or more of reducing agent.

The reaction times and procedures will vary with the reactivity of the aldehyde. Typically, the bound aldehyde is contacted with the amine, preferably in the presence of the dehydrating agent. The reaction mixture is shaken or stirred vigorously for 30-60 minutes and then the reducing

agent is added, preferably with additional dehydrating agent. Shortly after the reducing agent is added, the reaction is worked-up about 1% acetic acid.

The reductive amination reaction can be followed by ninhydrin test or the Kaiser test. However, it should be noted that the preformed imines also give a red or brown color in the ninhydrin test and some are quite stable to hydrolysis therefore falsely indicating a completed reaction. Samples for the Kaiser test should be heated at least for 15 minutes.

B. Formation of Diketopiperazines

The bound dipeptide can be prepared from the bound first amino acid derivative and the second amino acid derivative as described above. To initiate the formation of the diketopiperazine ring system, the protecting group, if present, on the terminal amino group of the dipeptide must first be removed. Standard conditions known in the art can be used to effect this deprotection. For example, the α-amino protecting group of the bound dipeptide **5** can be removed as described above, *e.g.*, in a 30% solution of piperidine in DMF.

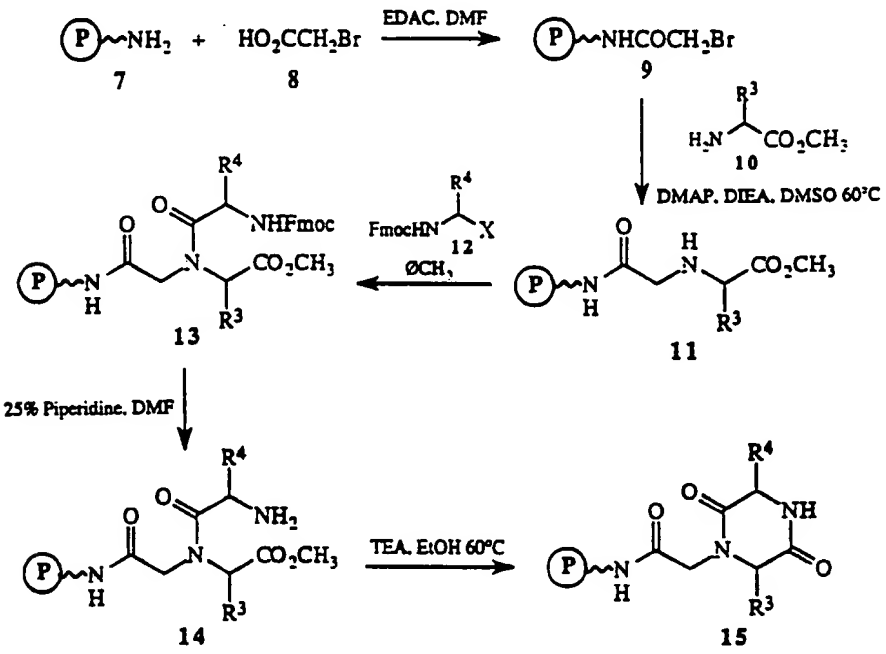
Upon removal of the terminal amino protecting group, cyclization occurs with the addition of acid or base and optionally heating the resin containing solution. As noted earlier a wide variety of acid, base and neutral cyclization conditions may be employed. Acidic cyclization conditions include, but are not limited to, methanol and acetic acid. Basic cyclization conditions include, but are not limited to, 0.1M piperidine or pyridine in DMF or methanol and TEA. Cyclization may also be performed under neutral conditions, as described above.

Formation of the product can be followed using the well-known Kaiser ninhydrin test for peptides or by a picric acid assay[19]. Alternatively, the progress of the reaction may be followed by gel phase ^{13}C NMR if at least one carbon atom of the dipeptide is the ^{13}C isotope. Amino

acids containing such isotopes are available commerically (*e.g.*, from New England Nuclear).

IV. Alternative Route to N-linked Diketopiperazines

An alternative route to bound diketopiperazines is illustrated in a preferred embodiment shown in Reaction Scheme II below. R^3 and R^4 are defined as R^1 and R^2 above and X is a carboxyl group or activated carboxyl group, such as an activated ester.



Reaction Scheme II

A. The First Bound Amino Acid Derivative

According to this embodiment, an amine functionalized resin **7**, such as TentaGel NH_2, is used. The terminal amino group is then reacted with bromoacetic acid **8**, typically in the presence of a coupling catalyst as described above to provide the resin-bound bromoacetamide **9**. A preferred coupling reagent is 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) in DMF. Preferably between 1 and 5 equivalents of EDAC are used. The bound bromoacetamide is further reacted with an

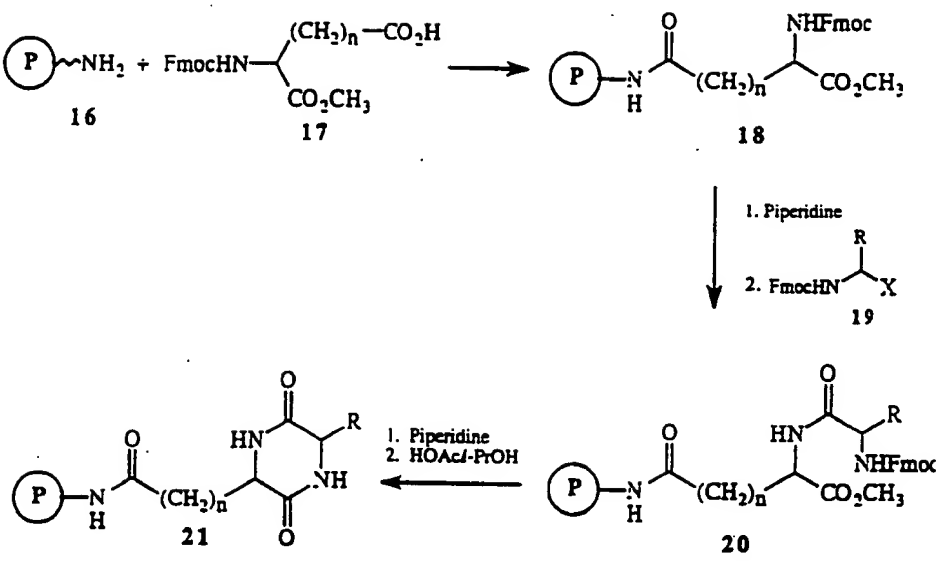
amino acid derivative **10** to form the bound amine **11**. Typically, the carboxyl group of the first amino acid will be protected during this displacement reaction. A preferred carboxyl group is the methyl ester, although other protecting groups known in the art will also work. Generally, the displacement reaction is conducted in a polar, aprotic and non-nucleophilic solvent such as dimethylsulfoxide (DMSO), often in the presence of a non-nucleophilic, exogenous base such as diisopropyl-ethylamine, dimethylaminopyridine or tetramethylpiperidine, at a temperature between room temperature and reflux. Preferred conditions include DMSO solution containing DMAP and DIEA and a reaction temperature of 60°C.

B. Preparation of the Diketopiperazine

The support-bound first amino acid **11** is then treated with a second amino acid as described above. The coupling reaction generally is performed in a non-polar, aprotic solvent such as toluene (ϕCH_3). Removal of the amino protecting group can be accomplished as described above. Typically, if the amino protecting group is an Fmoc group, it can be removed with 25% piperidine in dimethylformamide. The resulting dipeptide **14**, can be cyclized to the diketopiperazine **15** under basic or acidic conditions as described above. Preferably, the cyclization reaction is conducted with triethylamine in ethanol (EtOH) at 60°C or 0.1M acetic acid in ^{i}PrOH.

V. Preparation of C-Linked Diketopiperazines

A further embodiment of the present invention involves the preparation of C-linked diketopiperazines, *i.e.*, those linked to the support through the α-carbon atom of the first amino acid derivative. A specific embodiment of this method is shown below in Reaction Scheme III. R and X are defined as R^1 and X above, respectively.



Reaction Scheme III

A. The First Bound Amino Acid

According to this embodiment, an amino derivatized support **16**, such as TentaGel S NH_2 or Pharmacia Mono A is used. The support is then treated with a suitably protected carboxyalkyl amino acid **17**, wherein n is an integer between 0 and 6 and n is 0 is a valence bond. Preferably, a lower alkyl ester is used. Typically, the amino group and the carboxyl group of the sidechain of the amino acid **17** will be protected. Preferred amino protecting groups include Fmoc. Preferred carboxyl protecting groups include lower alkyl esters including the methyl ester illustrated.

B. Formation of the Diketopiperazine

Deprotection of the amino group, *e.g.*, with piperidine, yields the free amino group which can then be reacted with a suitably protected amino

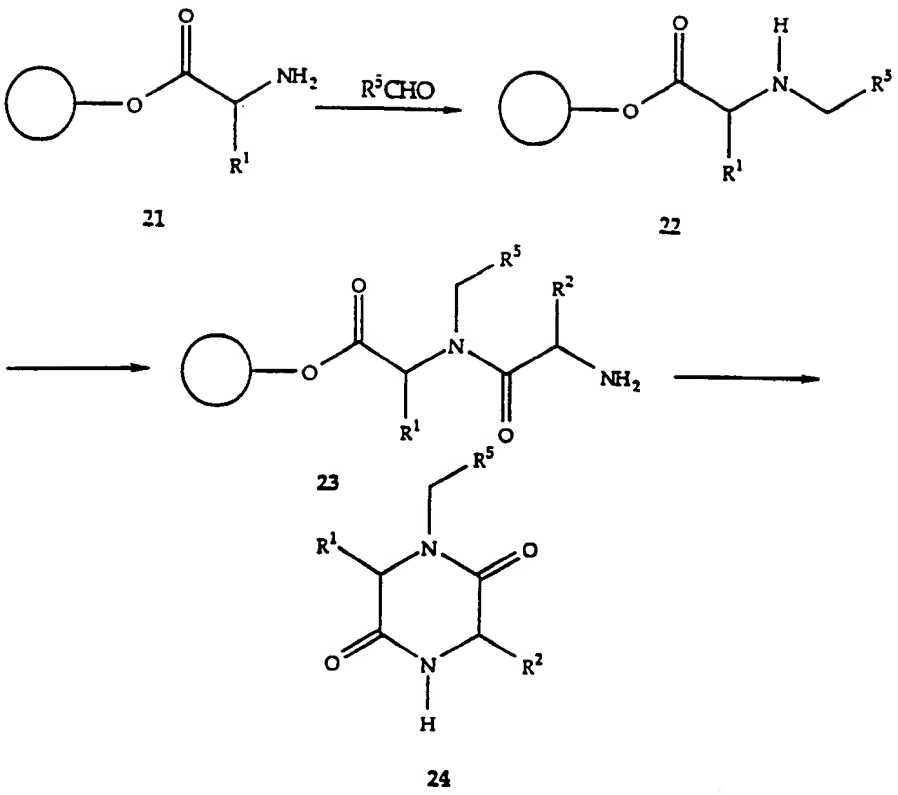
acid derivative 19 under conditions effective to form the bound dipeptide 20. The conditions for producing the dipeptide described above with regard to N-linked diketopiperazines can also be used to yield dipeptide 20. With certain steric amino acids, coupling of the second amino acid to the resin bound first amino acid proceeds more effeciently using HATU and a solvent mixture of DMF and methylene chloride.

As discussed above, treatment of the dipeptide, 20, with either acidic or basic conditions yields the diketopiperazine. According to a particularly preferred embodiment, the cyclization is performed under basic conditions, such as with piperidine.

In another embodiment, the cyclization is conducted in methanol. Since the starting material is typically recovered from HPLC as the TFA salt, an acid catalyzed reaction can be assumed when using methanol. Such conditions, however, provide for faster cyclization reactions.

VI. Preparation of Soluble N-Alkylated Diketopiperazines

A further embodiment of the present invention provides for the synthesis of N-alkylated diketopiperazines as shown in Reaction Scheme IV below. R^1 and R^2 are as above. R^5 is alkyl, aryl, heteroaryl, aralkyl, or heteroaralkyl. According to this embodiment, initial peptide synthesis occurs on a solid support and is followed by concomitant cyclization and cleavage from the support to yield soluble N-alkylated diketopiperazines.



Reaction Scheme IV

A. The Bound First Amino Acid Derivative

A first amino acid is coupled, via its carboxy terminus to a support. Typically, a support having hydroxyl groups at the surface will be utilized.

Suitable solid supports include Merrifield resin and hydroxy-PAM resin (available from Advanced Chemtech). In a preferred embodiment, a non-cleavable TentaGel hydroxy resin is used.

Generally, the amino group of the amino acid will be protected with a suitable protecting group during the coupling reaction. A variety of coupling catalysts can be used to effect the attachment of the amino acid, as described above. Preferably, the coupling is conducted in the presence of DMAP and DIC. Typically, between about 0.1 and about 0.5 equivalents of DMAP or between about 5-10 equivalents of DIC, based on resin loading, will be used.

B. The Reductive Amination

The amino group of the bound first amino acid is then alkylated. For example, the bound first amino acid can be treated with conventional alkylating agents, such as R^5CH_2X, where X is bromine or iodine. However, care should be taken to prevent over alkylation if this approach is attempted.

In a preferred embodiment, the bound first amino acid is treated with an aldehyde of formula R^5CHO where R^5 is alkyl, aryl, heteroaryl, aralkyl, or heteroaralky. (One of skill in the art will appreciate that any protecting groups present on the amine group of the bound first amino acid should be removed prior to the reductive amination reaction.) Preferably, the reductive amination is performed in the absence of acid and in the presence of a dehydrating reagent, as standard solid phase reaction conditions[20] (aldehyde, sodium cyanoborohydride, 1% acetic acid in DMF) resulted in over alkylation of the amino acid and yielded the corresponding N,N-dialkyl amino acid. Preferred dehydrating agents include molecular sieves, magnesium sulfate, sodium sulfate, trimethyl orthoformate, zinc chloride, and the like. More preferably, the dehydrating agent is in a form which can be easily washed away from the solid support or is even used as

the solvent. Most preferably, the dehydrating agent comprises trimethylorthoformate.

Typically, an excess of both the aldehyde and the reducing agent is used. Preferably, at least 5 equivalents of aldehyde and/or at least 5 equivalents of reducing agent is used. More preferably, the alkylation is performed with about 10 equivalents or more of aldehyde and about 10 equivalents or more of reducing agent.

The reaction times and procedures will vary with the reactivity of the aldehyde. For example, with short alkyl aldehyde with little steric hindrance (e.g., propanal or isovaleraldehyde), the bound first amino acid derivative is contacted with the aldehyde, preferably in the presence of the dehydrating agent. The reaction mixture is shaken or stirred for 30-60 minutes and then the reducing agent is added, preferably with additional dehydrating agent. For alkyl aldehydes with more steric hindrance (e.g., heptanal, t-butylacetaldehyde, and cyclohexane carboxaldehyde), the above procedure is followed. However, shortly after the reducing agent is added (e.g., 10 minutes), the reaction is worked-up with a dilute solution of a protic solvent, preferably a 5% or less solution of a lower alkyl alcohol in trimethyl orthoformate, and more preferably about a 1% aqueous solution of methanol in trimethyl orthoformate. For aromatic and sterically hindered alkyl aldehydes (e.g., benzaldehyde, pivaldehyde, and 2-ethylbutanal), the procedure for short alkyl aldehydes is followed. However, a dilute solution of a weak acid, preferably a 5% or less solution of acetic acid in trimethyl orthoformate, more preferably about a 1% solution of acetic acid in trimethyl orthoformate, is added immediately after the addition of the reducing agent.

The reductive amination reaction can be followed by ninhydrin test or the Kaiser test. However, it should be noted that the preformed imines also give a red or brown color in the ninhydrin test and some are quite stable to hydrolysis therefore falsely indicating a completed reaction. Samples for the Kaiser test should be heated at least for 15 minutes.

C. Formation of the Bound Dipeptide

The secondary acylation reaction to yield the dipeptide may be performed using a variety of coupling catalysts, as described above. In a preferred embodiment, the coupling catalyst will comprise PyBrOP, BOPCl, HATU, HOAt (1-hydroxy-7azabenzotriazole *see* Carpino (1993) J. Am. Chem. Soc. 115:4397-4398), DCC, or other coupling catalysts used for making amide bonds. Preferably, HATU is used. Typically, the ratio of free amino acid to coupling catalyst is from about 1:1 to 1:10, more preferably from about 1:1 to 1:5, and most preferably, about 1:1.

Often the secondary acylation reaction is performed in the presence of an exogenous base. Preferably a slight excess of base is used. The ratio of free amino acid to exogenous base will range from about 1:1.1 to about 1:10, preferably from about 1:1.1 to about 1:5 and more preferably from about 1:1.1 to about 1:3. In a particularly, preferred embodiment, the exogenous base will be soluble in the reaction solvent. Particularly preferred exogenous bases include tri(lower alkyl)amines, such as diisopropylethylamine (DIEA) or triethylamine (TEA).

Typically, a polar, aprotic solvent, such as dichloromethane, is used. If solubility of the amino acid proves problematic, it is often desirable to use a mixture of solvents, for example a 1:1 mixture of dichloromethane and DMF.

It should be noted that care should be taken when deprotecting reactive functionalities of the bound dipeptide prior to the cyclization reaction. As many protecting groups are cleaved by treatment with acid, triethyl ammonium trifluoroacetate often is produced. A basic wash with diluted DIEA to remove the excess acid may release considerable amounts of diketopiperazine.

D. Formation of the N-Alkylated Diketopiperazine

Treatment of the N-alkylated dipeptide with either acid (for example, 1% acetic acid) or base (for example, 1% triethylamine) in an

inert solvent, preferably toluene, methanol or DMSO, affords the desired N-alkylated diketopiperazine.

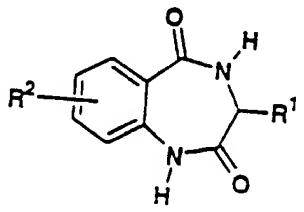
VII. Preparation of Homo-Diketopiperazines

One of skill in the art will readily appreciate that if either the first or second amino acid derivative comprises a beta-amino acid derivative rather than an alpha-amino acid derivative, then homo-diketopiperazines having a 7-membered ring can be produced. Many beta-amino acids are commercially available, for example from Aldrich Chemical Co., Milwaukee, Wisconsin and Bachem Biosciences, Philadelphia, PA.

Beta-substituted beta amino acids can be readily prepared via the Arndt-Eistert reaction with the corresponding alpha-substituted alpha amino acid (i.e., treatment with oxalyl chloride, followed by diazomethane). Conditions for effecting this transformation are described in Patai "The Chemistry of Diazonium and Diazo Compounds", Wiley, NY (1978) pp. 593-644; Chaturredi *et al.* (1970) J. Med. Chem. 13:177 and Marini *et al.* (1992) Synthesis 1104 (1992).

Chiral alpha-substituted beta amino acids can be prepared as shown in Figure 2. The carboxylic acid intermediate 27 can be prepared using Evans methodology. *See* Evans *et al.* J. Amer. Chem. Soc. 104:1737 (1982). Curtius rearrangement, followed by hydrolysis and deprotection provides the desired alpha-substituted beta amino acid. *See* Banthorpe, in Patai, "The Chemistry of the Azido Group", pp. 397-405, Interscience, New York (1971); Pfister and Wyman (1983) Synthesis 38.

In a preferred embodiment, the beta amino acid comprises anthranilic acid, isatoic anhydride, or a substituted anthranilic acid to afford benzodiazepine-1,4-diones of the formula:



An alternative synthesis for homo-diketopiperazines can be found in Figure 3. R^1 and R^5 are as defined above. 2-Amino-4-hydroxybenzoic acid is immobilized on a solid support via the hydroxy residue. Treatment with an aldehyde of formula R^5CHO and intramolecular trapping of the imine intermediate affords the bicyclic compound **30**. Reduction yields the corresponding N-alkylated compound. Treatment with phosgene and base, followed by alkylation with amino acid derivative **33** yields the homo-diketopiperazine.

VIII. Chelating Groups

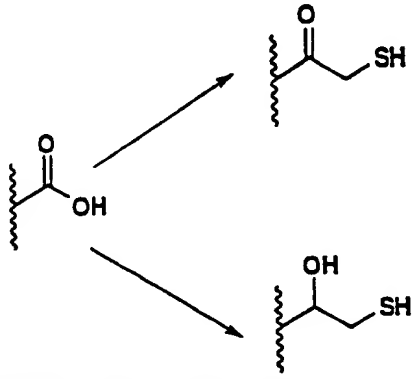
According to a preferred embodiment of the present invention, a chelating group is introduced as a substituent off of the diketopiperazine ring. As discussed above, the chelating group, optionally in a protected form, may be present throughout the preparation of the diketopiperazine ring skeleton and can be introduced with either the first or second amino acid. In a preferred embodiment, the chelating group will comprise a carboxylate, malonate, hydroxamate, or thiolate group, such as a mercapto alcohol or a mercapto ketone.

Other embodiments provide for post-cyclization transformation of the carboxyl group to yield alternative chelating groups. For example, the carboxyl group can be converted to the corresponding hydroxamate. In general, hydroxamates are obtained by treating a carboxylic acid with either hydroxylamine or an O-protected hydroxylamine. Typical O-protected hydroxylamines include t-$BuONH_2$, $THPONH_2$, and t-BDMS-ONH_2 where t-Bu represents the corresponding t-butyl ether, THP represents the corresponding tetrahydropyran ether, and t-BDMS represents the corresponding t-butyldimethylsilyl ether. Alternatively, treatment of the corresponding ester with hydroxyl amine yields the desired hydroxamate.

More specifically, the free base of hydroxylamine is generated by treatment of an excess of hydroxylamine hydrochloride with an excess of potassium or sodium hydroxide in a polar solvent, such as methanol. The solution is filtered and added to the O-benzyl or O-alkyl ester of the

carboxylic acid to yield the hydroxamic acid. Alternatively, an O-protected hydroxylamine is coupled to the acid using EDC and DIEA in a nonpolar solvent, such as dichloromethane. If the acid is not soluble in dichloromethane, DMF may be added. The protecting group, if present, can then be removed using standard deprotection conditions, as known in the art.

In a particularly preferred embodiment, the carboxylic acid group is converted to a mercapto alcohol or ketone as shown below:



Procedures for effecting this transformation can be found in co-pending application U.S. Serial Number 08/329,420, filed October 27, 1994 which is incorporated herein by reference for all purposes.

IX. Polycyclic Structures Based on Diketopiperazines

The diketopiperazines disclosed herein include those where the diketopiperazine forms one or more rings in a polycyclic structure. Such structures are readily formed by employing a heterocycle containing a secondary amine structure in the cyclic backbone. This amine is synthetically employed in the manner described above and results in a polycyclic product either resin bound or which after cleavage is in soluble form.

Examples of such polycyclic structures are set forth in Figures 8 and 9. Specifically, in Figure 8, soluble 2-carboxyl-4-aminopiperidine 44 having both amino groups blocked with different orthoganol protecting groups is bound to bound to a solid support in the manner described herein

to provide for compound 45. Orthogonal deprotection removes the first protecting group while retaining the second followed by acylation to provide for acylated-4-amino piperidine having a protecting group on the ring amino group, compound 46. Removal of this protecting group followed by coupling of an Fmoc protected amino acid (R_2 is the amino acid side chain) provides for compound 47. Removal of the Fmoc group followed by acid catalyzed cyclization provides for fused heterocyclic compound 48 which is a polycyclic diketopiperazine compound. It is understood, of course, that the Fmoc group is shown for illustrative purposes and that other blocking groups can be used or, alternatively, the amine need not be protected depending upon the reaction conditions.

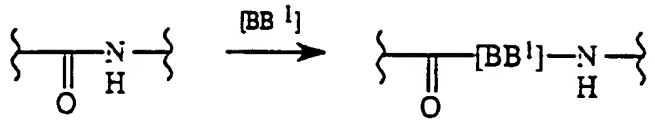
Figure 9 illustrates similar chemistry as set forth in Figure 8 with the exception that a 5 member proline ring is employed rather than the piperidine ring. Specifically, soluble 4-amino proline 49 having both amino groups blocked with different orthoganol protecting groups is attached to a solid support in the manner described above to provide for compound 50. Alternatively, compound 50 is formed attached to the support and the reaction scheme is continued.

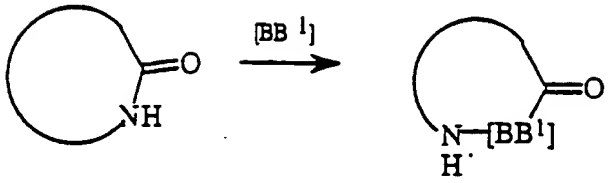
Orthogonal deprotection removes the first protecting group while retaining the second followed by acylation to provide for acylated-4-amino proline having a protecting group on the ring amino group, compound 51. Removal of the protecting group followed by coupling of an Fmoc protected amino acid (R_2 is the amino acid side chain) provides for compound 52. Removal of the Fmoc group followed by acid catalyzed cyclization provides for fused heterocyclic compound 53 which is a derivative of the original pyrrolidinyl group. It is understood, of course, that the Fmoc group is shown for illustrative purposes and that other blocking groups can be used or, alternatively, the amine need not be protected depending upon the reaction conditions.

X. Post-Cyclization Transformations

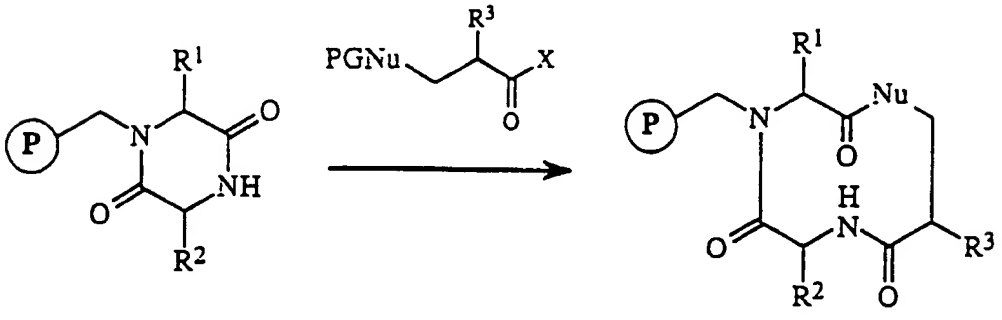
A wide variety of post-cyclization transformations are also known.[21 22] For example, one or both of the ring nitrogens may be alkylated or acylated. Acylation or alkylation is often an useful step in forming multiple ring systems which contain the diketopiperazine structure. For example, alkylation or acylation of either or both of the R^1 or R^2 substituents may yield a ring with a nitrogen of the diketopiperazine ring adjacent the carbon to which the substituent is attached. Acylation of the amide nitrogen, followed by nucleophilic attack by the acyl group on the adjacent carbonyl may be employed to synthesize cyclols, azacyclols or thiacyclols. The C-2 and C-5 carbons may also be transformed into dithiones. Alternatively, the ring system may be oxidized to form the corresponding heteroaromatic ring, or the carbonyls may be selectively reduced. The amide units of the diketopiperazines may also be converted into mono- or bis-lactim ethers as described above. Other transformations will be apparent to those of skill in the art.

An example of the extension of the methods of the invention to synthesize other compounds containing the diketopiperazine structure involves the alkylation or acylation of one or both of the diketopiperazine amide nitrogens with a building block ([BB^1]) containing a nucleophile, and the subsequent insertion of the building block into the diketopiperzine structure. This is illustrated generally below. Typical building blocks include molecules that have an electrophilic moiety which is reactive toward either or both of the amide nitrogen atoms, such as an activated carboxyl group, in addition to a nucleophilic moiety, such as amine or hydroxyl group.

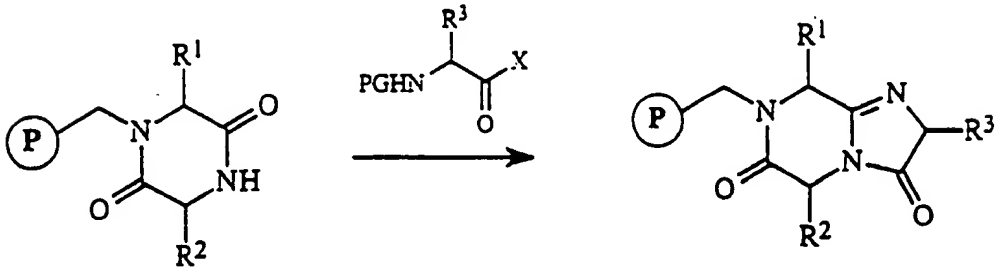


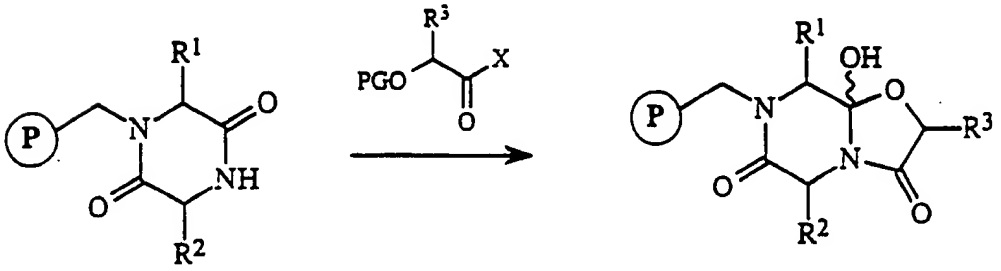


For example, acylation at an amide nitrogen with an molecule comprising a protected nucleophilic moiety (PGNu), such as protected nitrogen, oxygen or sulfur, followed by deprotection of the nucleophile and rearrangement, leads to expansion of the diketopiperzine ring by the insertion shown below. This provides a method for the synthesis of cyclols, acylamidines or expanded cyclic peptides.

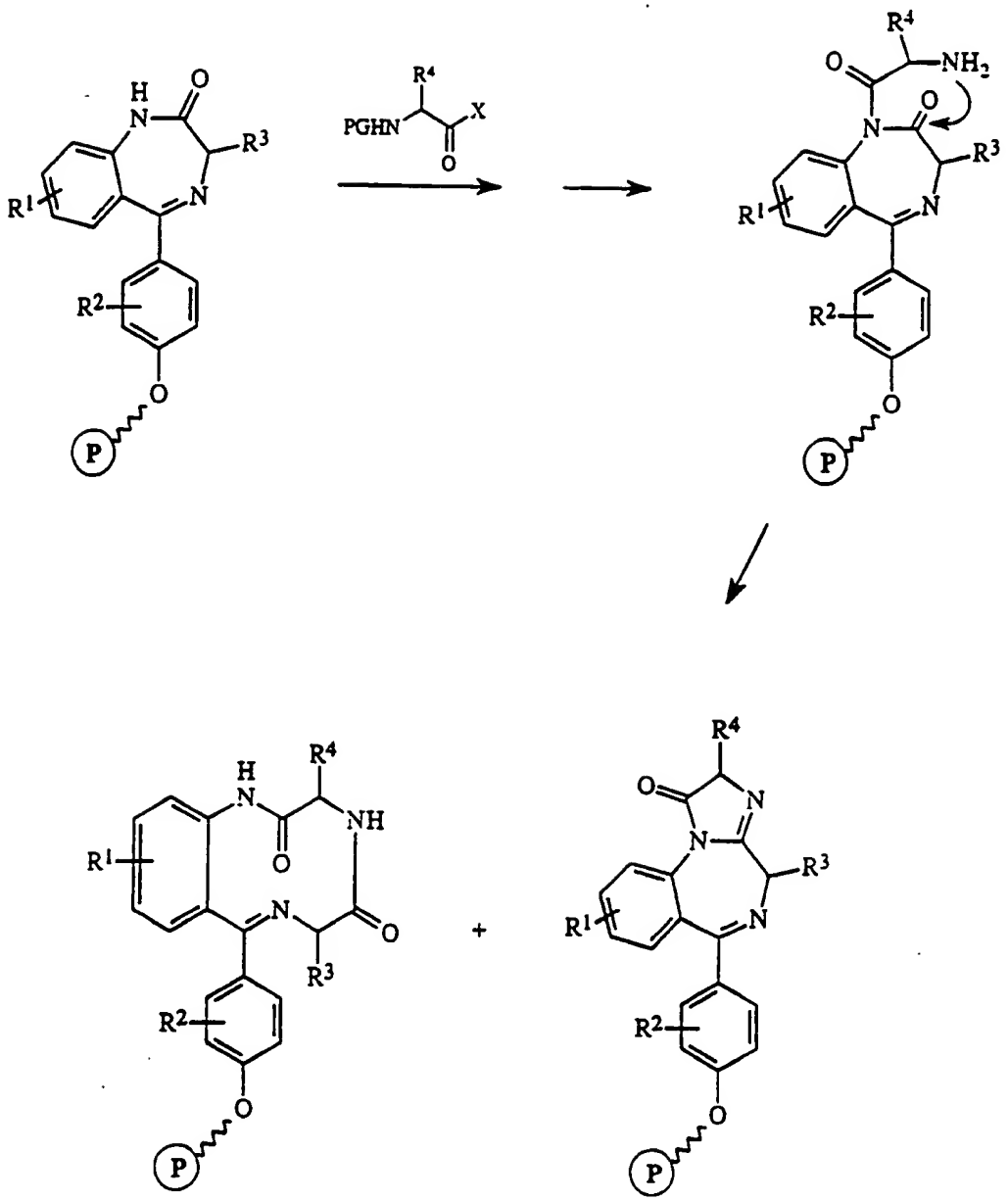


By varying the reaction conditions, insertion of an amino acid or hydroxy acid, followed by nucleophilic attack on the adjacent carbonyl and, in the case of an amino acid, the elimination of water, leads to 6:5 fused ring systems, as shown below. It will be appreciated that, under suitable conditions, coupling without additional cyclization or expansion may be performed. Such coupling allows for the formation of diketopiperazine chains or insertion of diketopiperazines into peptide chains.

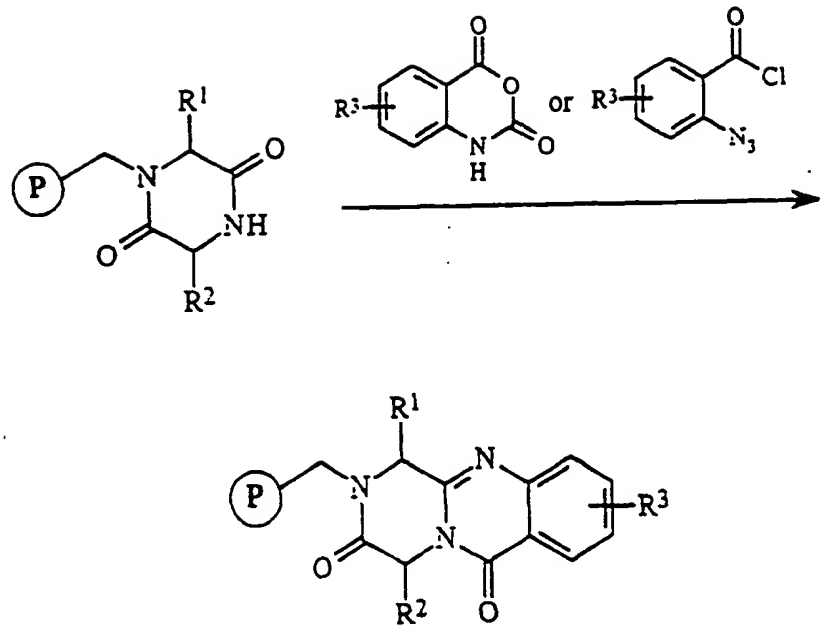




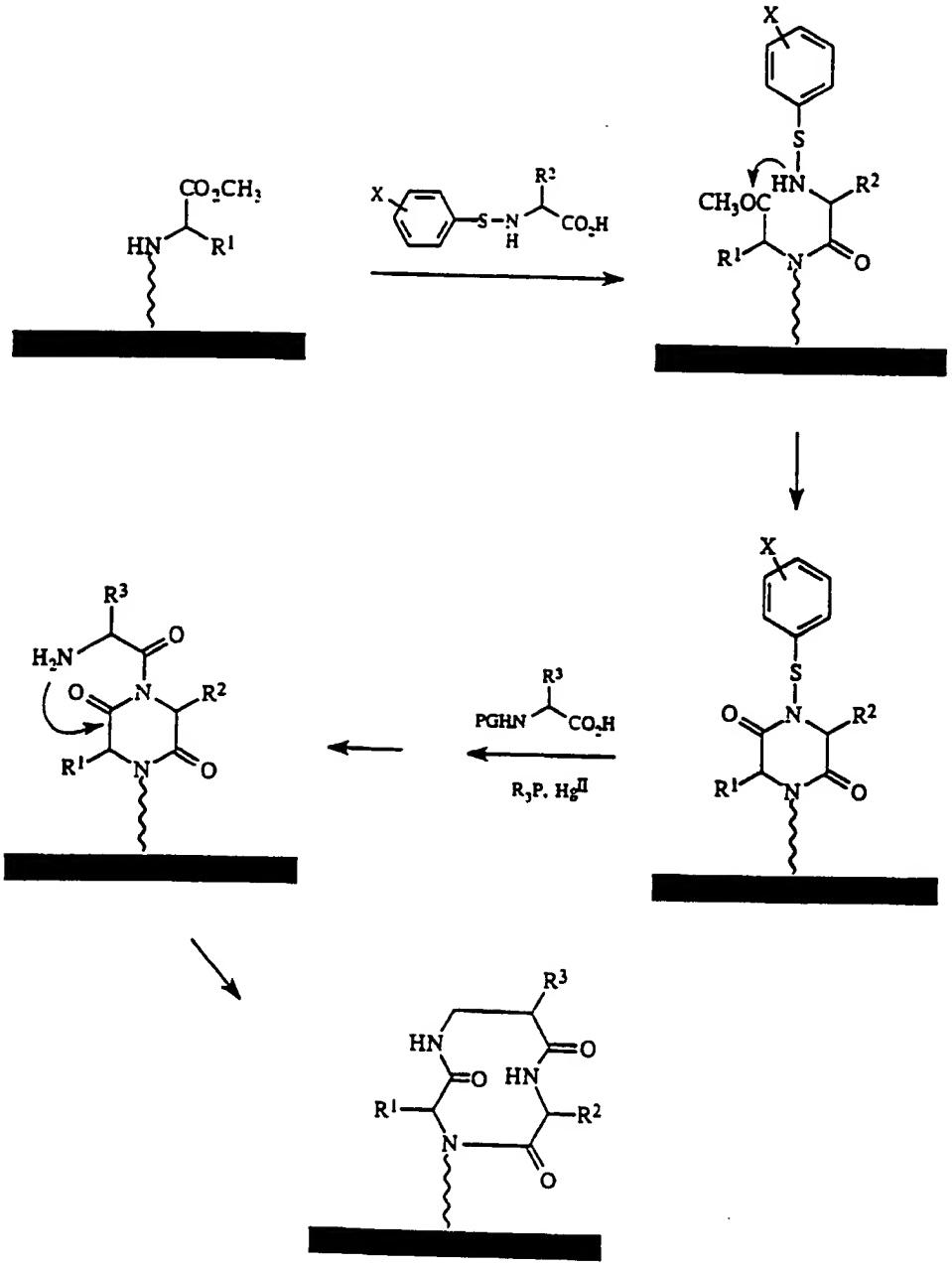
One application of this approach with important utility is the formation of benzodiazepine derivatives. Reaction of bound benzodiazepines with Fmoc-protected amino acids provides the bound N-acylated derivative as shown. Removal of the protecting group allows for formation of the cyclol or a 7:5 fused ring system by the mechanisms just described.



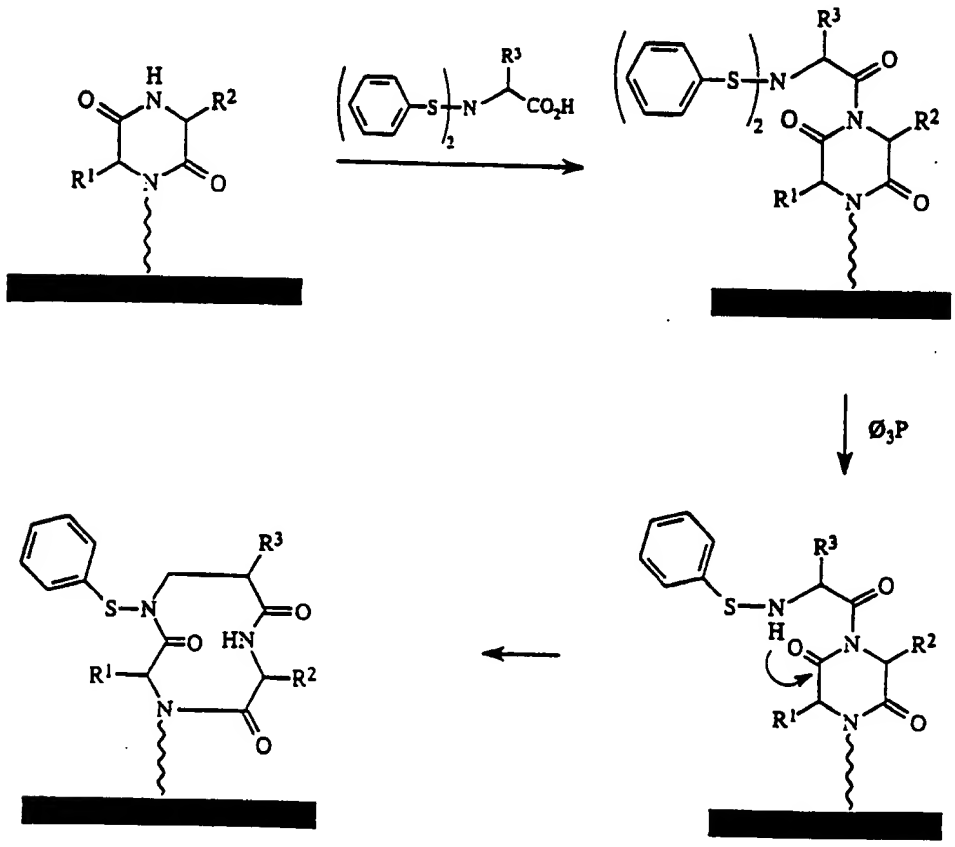
Insertion of anthranylates can be used to produce quinazolines.



The above-described extensions can be approached in an iterative fashion. Two examples of this approach are shown below. In the first approach, a bound amino acid is coupled with a phenylthioamine derivative of a second amino acid to form a dipeptide which is attached to the support at the amide nitrogen (X represents any substituent). This is cyclized to form an N-phenylthiodiketopiperazine. Removal of the phenylthio substituent, followed by coupling of a third amino acid provides the amidodiketopiperazine shown. Deprotection of the primary amine allows the amine to attack the adjacent carbonyl carbon to form the cyclol as shown.


X
S
HN
R2
CH3OC
R1
N
O
CO2CH3
HN
R1
X
S
N
H
R2
CO2H
R3
H2N
O
N
R2
O
R1
N
O
R3
PGHN
CO2H
R3P. HgII
HN
O
HN
R1
R2
N
O

Alternatively, the third amino acid derivative may be a N,N-phenylthio derivative. Coupling of the amino acid to the diketopiperazine, followed by reaction with triphenylphosphine to remove one of the phenylthio substituents, allows expansion to the cyclol as shown.



In addition to the above post-cyclization transformations, Figure 7 illustrates solid phase transformation leading to formation a polycyclic structure based on C-linked diketopiperazine. Specifically, soluble 2-carboxyl diketopiperazine 39 having both amino groups blocked with different orthoganol protecting groups is attached to a solid support in the manner described above to provide for compound 40. Alternatively, compound 40 can be formed attached to the support and the reaction scheme is continued.

Orthogonal deprotection removes the first protecting group while retaining the second followed by acylation to provide for acylated-4-amino diketopiperazine having a protecting group remaining on the 1-amino group, compound 41. Removal of this protecting group followed by coupling of an Fmoc protected amino acid (R_3 is the amino acid side chain) provides for compound 42. Removal of the Fmoc group followed by acid catalyzed cyclization provides for fused heterocyclic compound 43 which is a polycyclic derivative of the original diketopiperazine group. It is understood, of course, that the Fmoc group is shown for illustrative purposes and that other blocking groups can be used or, alternatively, the amine need not be protected depending upon the reaction conditions.

XI. Libraries of Diketopiperazines

In a preferred embodiment of the present invention, the above described solid phase synthesis is adapted to the formation of a library of diverse diketopiperazine structures, comprising a plurality of polymer beads having a plurality of surface-bound diketopiperazines. The diketopiperazines bound to each of said beads are substantially homogeneous and have a composition different from diketopiperazines bound to selected other beads. In a preferred embodiment, tags, more preferably oligonucleotide tags, are also affixed to the beads identifying the diketopiperazines. In a still more preferred embodiment, diketopiperazines and tags may optionally be cleaved, *e.g.*, to facilitate detection or to provide a soluble library.

These libraries will be referred to herein as Encoded Synthetic Libraries ("ESL") and are described generally in co-pending U.S. Patent Applications Serial Nos. 08/149,675, 08/146,886, 07/946,239 and 07/762,522, the full disclosures of which are incorporated herein by reference. Such libraries can be screened to isolate individual oligomers that bind to a receptor or possess some other desired property.

A general method for synthesizing such collections of diketopiperazines typically involves a random combinatorial ("stochastic") approach and the chemical and/or enzymatic assembly of amino acid monomer units. One process for producing libraries of N- or C-linked diketopiperazines comprises the steps of: (a) binding first amino acid derivatives to beads, wherein the amino acid derivatives bound on individual beads are substantially homogeneous and have a composition different from amino acid derivatives on selected other beads; (b) reacting the bound first amino acid derivatives with a plurality of second amino acid derivatives to form a plurality of dipeptide derivatives bound on individual beads that are substantially homogeneous and have a composition different from dipeptide derivatives on selected other beads; and (c) cyclizing the bound dipeptide derivatives to form a plurality of beads having diketopiperazines bound thereon, wherein the diketopiperazine derivatives bound to each bead are substantially homogeneous and have a composition different from diketopiperazines on selected other beads.

Libraries of N-alkylated diketopiperazines can also be obtained by a similar process comprising the steps of: (a) binding first amino acid derivatives to beads, wherein the amino acid derivatives bound on individual beads are substantially homogeneous and have a composition different from amino acid derivatives on selected other beads; (b) reacting the bound first amino acid derivatives with a plurality of aldehydes and a reducing agent; (c) reacting the bound N-alkylated amino acid with a plurality of second amino acid derivatives to form a plurality of dipeptide derivatives bound on individual beads that are substantially homogeneous

and have a composition different from dipeptide derivatives on selected other beads; and (c) cyclizing the bound dipeptide derivatives with concomitant cleavage of the compounds from the beads to form a plurality of N-alkylated diketopiperazines.

The steps outlined in either process above may be optionally followed by steps of pooling and/or apportioning the beads among a plurality of reaction vessels or by forming a heterogeneous mixture of beads. Oligonucleotide tag components may be optionly attached to the beads before, during or after each of steps (a)-(c) as described below. Typically, substantially equal numbers of solid supports will be apportioned to each reaction vessel. Those of skill in the art will recognize that the same chemical building block can be employed in different coupling steps and that the same chemical building block can be employed in more than one coupling reaction (reaction vessel) of a single coupling step.

The identifier tag has a recognizable feature that is, for example, microscopically or otherwise distinguishable in shape, size, mass, charge, or color. This recognizable feature may arise from the optical, chemical, electronic, or magnetic properties of the tag, or from some combination of such properties. In essence, the tag serves to label a molecule and to encode information decipherable at the level of one (or a few) molecules or solid supports. By using identifier tags to track the synthesis pathway that each member of a chemical library has taken, one can deduce the structure of any chemical in the library (*i.e.*, the sequence of monomers of any oligomer) by reading the identifier tag.

The identifier tags identify each monomer coupling or other reaction step that an individual library member or solid support has experienced and record the step in the synthesis series in which each amino acid was added or other chemical reaction performed. The tags may be attached immediately before, during, or after the amino acid addition or other reaction, as convenient and compatible with the type of identifier tag, modes of attachment, and chemistry of diketopiperazines or other molecular

synthesis. The identifier tag can be associated with the diketopiperazines through a variety of mechanisms, either directly, through a linking molecule, or through a solid support upon which the oligomer is synthesized. In the latter mode, one could also attach the tag to another solid support that, in turn, is bound to the solid support upon which the oligomer is synthesized. The identifier tag is added when the solid supports that have undergone a specific monomer addition or other chemical reaction step are physically together and so can be tagged as a group, *i.e.*, prior to the next pooling step.

One can construct microscopically identifiable tags as small beads of recognizably different sizes, shapes, or colors, or labeled with bar codes. The tags can be "machine readable" luminescent or radioactive labels. The identifier tag can also be an encodable molecular structure. The information may be encoded in the size (the length of a polymer) or the composition of the molecule. Perhaps the best example of this latter type of tag is a nucleic acid sequence, *i.e.*, RNA or DNA assembled from natural or modified bases. The tag can also comprise a variety of light-addressable molecules, such as fluorescent or phosphorescent compounds, the spectral properties of which can be changed (*e.g.* by photobleaching) and therefore used to store information. In one such mode, a bead incorporates a variety of fluorophors, each of which can be selectively photobleached, and so rendered incapable of fluorescence or of diminished fluorescence. During each coupling or chemical reaction step, the bead is irradiated (or not) to photobleach (or not) one or more particular types of fluorophors, thus recording the monomer identity in the oligomer synthesized.

Synthetic oligodeoxyribonucleotides are especially preferred information-bearing identifier tags. Oligonucleotides are a natural, high density information storage medium. The identity of monomer type and the step of addition or any other information relevant to a chemical synthesis procedure is easily encoded in a short oligonucleotide sequence.

Oligonucleotides, in turn, are readily amenable for attachment to a wide variety of solid supports, oligomers, linkers, and other molecules. For example, an oligonucleotide can readily be attached to a peptide synthesis bead.

Of especial advantage to using oligonucleotide tags is the ability to achieve tremendous levels of target amplification through the polymerase chain reaction (PCR),[23] and other nucleic acid replication and amplification techniques. Although the most commonly used *in vitro* DNA amplification method is PCR, suitable alternate amplification methods include, for example, nucleic acid sequence-based amplification, amplified antisense RNA, and the self-sustained sequence replication system. Only tiny quantities (with highly selective and efficient methods, even a single copy is sufficient) of DNA template is required for PCR, enabling one to use solid supports of microscopic dimensions and obtain larger libraries.

Libraries of N-alkylated diketopiperazines have been produced using the procedures described above. A first library was prepared using two first amino acids (i.e., D-Asp and L-Asp). Each of the separate first amino acid derivatives were subjected to reductive alkylation with a mixture of 9 aldehydes, as shown in Figure 4. The beads were then mixed. Coupling of 8 different second amino acid derivatives and cyclization yielded 16 libraries with 9 members each (or 144 different diketopiperazines).

A second library of 864 diketopiperazines was constructed using L- and D-Glu and D-Asp and L-Asp as the first bound amino acid derivative. See Figure 5. The reductive alkylation was performed with 18 different aldehydes. The diketopiperazines were formed under acidic conditions. Optionally, after formation of the library, the carboxyl groups can be converted to hydroxamates as set forth below.

Preferably, the bead-bound diketopiperazines are synthesized using automated procedures and instrumentation such as described in co-pending U.S. Patent Applications Serial Nos. 08/149,675 and 08/146,886 incorporated herein by reference in their entirety. Briefly, the

instrumentation for generating synthetic libraries of tagged molecules requires plumbing typical of peptide synthesizers, together with a large number of reservoirs for the diversity of monomers and the number of tags employed and the number of simultaneous coupling reactions desired. The tag dispensing capability translates simple instructions into the proper mixture of tags and dispenses that mixture. Monomer building blocks are dispensed, as desired, as specified mixtures. Reaction agitation, temperature, and time controls are provided. An appropriately designed instrument also serves as a multi-channel peptide synthesizer capable of producing 1 to 50 milligrams (mg) of crude product for up to 100 specific peptides for assay purposes (*see also* PCT patent publication 91/17823, incorporated herein by reference).

Typical instrumentation comprises (1) means for storing, mixing, and delivering synthesis reagents, such as peptide and oligonucleotide synthesis reagents; (2) a sealed chamber into which the various reagents are delivered and inside of which the various reactions can proceed under an inert atmosphere; (3) a matrix of sealed reaction vessels; (4) means for directing the flow of reagents to the appropriate reaction vessels: (5) means for combining and partitioning small (0.1-1000 μm) beads; and (6) means for washing the beads in each reaction vessel at the conclusion of each chemical reaction. The matrix of reaction vessels can have any one of several designs. For example, the vessels can be arranged in a circle so that the vessels can be made to rotate about a central axis (*i.e.*, a centrifuge). Alternatively the vessels can be arranged in a 12 x 8 matrix (96-well microtiter plate format). Any arrangement amenable to accessibility by robotic delivery, aspiration, and transfer functions is useful for some applications.

In some cases, the reactions employed are used relative to the system design. For example, in the reductive alkylation step, sodium cyanoborohydride has a low solubility in trimethylorthoformate (< 100 mg/mL) and precipitation is formed easily clogging lines and valves.

However, a dichloromethane/trimethylorthoformate mixture gave good results with this equipment. Instead of dissolving sodium cyanoborohydride in trimethylorthoformate, a 1M solution in THF (available from Aldrich Chemical Company, Milwaukee, Wisconsin) was used. The total amount of trimethylorthoformate with these solvents was reduced to one third.

The system used for combining and redistributing particles can have one of several designs. For instance, the beads can be suspended in a solvent of appropriate surface tension and density such that a robotic pipetting instrument can be used to transfer the beads to a combining vessel. After mixing, the beads can be redistributed to the reaction vessels by the same robotic pipettor. Alternatively, the beads can be combined by using a special valved reaction chamber. The valve is opened to allow solvent flow to transfer the beads to a combining vessel. After mixing, the beads are repartitioned by reversing the flow to each reaction vessel.

In another embodiment, the beads are combined using closely spaced reaction vessels with open top ends. Flooding the vessels allows the beads to mix. If the beads are magnetic, then the beads are re-partitioned by pulling the beads back down to the bottom of the vessels by application of a magnetic field. Non-magnetic beads are re-partitioned by vacuum suction through the bottom of the reaction vessels. In yet another embodiment, the beads may be partitioned by distributing them on a flat surface and then restricting them to certain sectors by covering them with a "cookie-cutter" shaped device.

The system for washing the beads can also have one of several designs. The beads can be washed by a combination of liquid delivery and aspiration tubing. Each reaction vessel has its own set of tubing, or a single set can be used for all reaction vessels. In the latter case, the liquid delivery and aspiration lines can be mounted on a robotic arm to address each vessel individually. The beads in each vessel can be made to form a single pellet by either centrifugation or the use of magnetic beads and application of a magnetic field. One can also employ a reaction vessel with

a bottom wall composed of a chemically inert membrane so that reagents can be removed from the vessels by application of a vacuum. Reagents can also be removed from each vessel by using vessels that can accommodate continuous flow through of reagents and washing solutions, *i.e.*, a vessel with luer fittings and membranes on each end.

In another embodiment, the library is formed using the Very Large Scale Immobilized Polymer Synthesis (VLSIPS™) technique, such as described in Pirrung, *supra*. The surface of the support comprises photoreactive protecting groups bound to functional groups on the support surface, *e.g.*, amine groups. These groups are removed from selected areas of the support surface by irradiation at an appropriate wavelength through a mask or filter. In an especially preferred embodiment, the irradiation is performed using photolithographic techniques such as those discussed in Pirrung, *et al.*, and copending U.S. Patent Application Serial Nos. 972,007, 805,727, 624,120, 954,646, 954,519, 850,356, 849,757, 492,462 and 362,901, each of which is incorporated herein by reference in its entirety. At least one first amino acid derivative is then bound to the selectively deprotected areas of the support surface. The first amino acid derivative may also include a photoreactive protecting group which may be removed at a wavelength of radiation the same as, or different from, the protecting groups used on the support surface.

Preferably a plurality of first amino acid derivatives are bound to the support surface by repeating the steps of deprotecting selected areas of the support surface and exposing the deprotected areas to a plurality of first amino acid derivatives at known locations on the support surface. The support is also irradiated to remove protecting groups from the first amino acid derivatives, whereupon, the support is contacted with at least one second amino acid derivative to form at least one dipeptide derivative. A plurality of second amino acid derivatives are reacted with the first bound peptide derivativesto form a plurality of dipeptide derivatives at known locations on the support surface. The dipeptide derivatives are then

cyclized to form a plurality of diverse N-linked or C-linked diketopiperazine structures at known locations on the support.

Alternatively, a plurality of amino acid derivatives are bound to known locations on a set of pins such as those described by Geysin[24], or Ellman.[25] These pins may be adapted to fit within individual reaction chambers, such as the wells of a microtiter plate, so that chemical reactions may be performed selectively at known pin locations. Thus, for example, a variety of first amino acid derivatives may be bound to known pin locations by placing the appropriate reagents in the individual wells of a microtiter plate and placing the appropriate pin in the desired well. Once the first amino acid derivatives have been bound, the pins are exposed selectively to wells containing an aldehyde, a second amino acid derivative or other reagents which are appropriate for performing the desired chemistry. Upon cyclization, a library of diverse diketopiperazine structures is formed.

XII. Screening of Diketopiperazine Libraries

The libraries of bound diketopiperazines may be screened for biological activity. Generally the library to be screen is exposed to a biological substance, usually a protein such as a receptor, enzyme, membrane binding protein or antibody, and the presence or absence of an interaction between the diketopiperazine and the biological substance is determined. Typically this will comprise determining whether the biological substance bound to one or more of the members of the library. Such binding may be determined by attaching a label to the biological substance. Commonly used labels include fluorescent labels such as fluorescein, phycoerythrin or Cy-3 (available from Molecular Probes, Eugene, OR). Other methods of labeling may be used, such as radioactive labels. The degree of binding affinity may be determined by quantitating the amount or intensity of the bound label. Thus, various lead compounds

may be selected by identifying which compounds bind the particular biological substance most effectively.

In a preferred embodiment, bead-based libraries are screened by assays in which each different molecule in the library is assayed for its ability to bind to a receptor of interest. The receptor is contacted with the library of diketopiperazines, forming a bound member between the receptor and any diketopiperazine in the library able to bind the receptor under the assay conditions. The bound diketopiperazine is then identified by examination of the tag associated with that diketopiperazine. The receptor to which the library is exposed under binding conditions can be a mixture of receptors, each of which is associated with an identifier tag specifying the receptor type, and consequently two tags are examined after the binding assay. Specific beads can be isolated in a receptor screening by a number of means, including infinite dilution, micromanipulation, or preferably, flow cytometry (*e.g.*, fluorescence activated cell sorting (FACS)). By adopting cell-sized solid supports or beads, one can use flow cytometry for high sensitivity receptor binding analysis and facile bead manipulation.

Diketopiperazines can be synthesized on beads and cleaved prior to assay. N- or C-linked diketopiperazines of interest can be cleaved from the beads to produce either untagged diketopiperazines in solution (the tag remaining attached to the bead) or tagged diketopiperazines in solution. Cleavage of the diketopiperazines from the beads may be accomplished using chemical, photocleavable or thermal systems. N-alkylated diketopiperazines are cleaved from the beads during cyclization.

In either event, the diketopiperazines of interest are cleaved from the beads but remain contained within the compartment along with the bead and the identifier tag(s). Oligonucleotides are preferred tags for such libraries, being readily PCR amplified and cloned into the commercially available TA cloning vector (Invitrogen, Inc.), a convenient form for storing tag information prior to analysis by DNA sequencing. In addition, oligonucleotide tags can be concatenated, as described in co-pending U.S.

Patent Application Serial No. 08/146,886, allowing one to collect pools of soluble tagged diketopiperazines, clone the concatenated tags from the selected pools, and then sequence the tags to identify the desired compounds.

Soluble tagged diketopiperazines can also be screened using an immobilized receptor. After contacting the tagged diketopiperazines with the immobilized receptor and washing away non-specifically bound molecules, bound, tagged diketopiperazines are released from the receptor by any of a wide variety of methods. The tags are optionally amplified and then examined and decoded to identify the structure of the molecules that bind specifically to the receptor. A tagged diketopiperazine in solution can be assayed using a receptor immobilized by attachment to a bead, for example, by a competition assay with a fluorescently labeled ligand. One may recover the beads bearing immobilized receptors and sort the beads using FACS to identify positives (diminished fluorescence caused by the library molecule competing with the labeled ligand). The associated identifier tag is then amplified and decoded.

In addition to identifying lead compounds, the nature of the binding between the diketopiperazines identified as having binding affinity to the biological substance may be studied by forming diketopiperazine derivatives based on the structure of the identified lead compound. These derivatives may include moieties and/or other structural alterations which produce steric and/or electronic perturbations in the structure of the lead compound. Screening this "library on a theme" against the biological substance and/or derivatives or mutants of the biological substance will yield useful information about the structural features important for biological activity. Such screening may also be performed under various conditions to determine the effects of solvent, agonists or antagonists, or temperature on binding. In addition, it will be appreciated that screening of diketopiperazine libraries will have utility in identifying diketopiperazines having novel and enhanced medicinal efficacy.

EXAMPLES

The following examples are for purposes of illustration only and are not intended to limit the scope of the invention in any manner.

Materials and Methods

Common reagents and solvents were purchased from Aldrich Chemical Company (Milwaukee, WI) or VWR Scientific. Amino acids were purchased from Aldrich, Sigma (St. Louis, MO), Bachem Bioscience, Inc. (Philadelphia, PA), Novabiochem (La Jolla, CA), SynPep or Peninsula Labs (Belmont, CA). BOP and Knorr linkers were purchased from Novabiochem. TentaGel resins were purchased from Rappe Polymere. PAL™ support(for peptide amides was purchased from Millipore, Inc.

Example 1

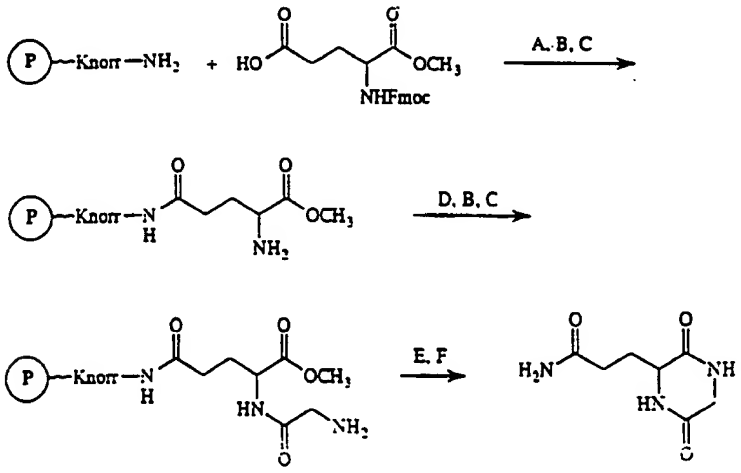
Synthesis of *cyclo*-GlnGly

The synthetic route to the named diketopiperazine is illustrated in Reaction Scheme IV, below. 580 milligrams (260 micromoles/gram (μmol/g)) of TentaGel S amine resin (Rappe Polymere) was washed with dimethylformamide (2 x 7 milliliters (ml)). *p*-[(R,S)-α-[1-(9H-fluoren-9-yl)-methyloxyformamido]-2,4-dimethoxybenzyl]phenoxyacetic acid (Fmoc-Knorr linker, 244 mg, 3 eq.), BOP (200 mg, 3 eq.), diisopropylethylamine (238 microliters (μl), 6 equivalents (eq.)) and DMF (6 ml) were added to the resin and the mixture was stirred for 30 minutes (min.) This coupling was performed twice on the resin. The resin was filtered and treated with acetic anhydride (Ac_2O)/piperidine (5ml) for 10 min., followed by three rinses with DMF (6 ml each). 30% piperidine/DMF (6 ml) was added to the resin and the resulting slurry was stirred for 20 min, to remove the Fmoc group.

The resin was next washed twice with ethyl alcohol and three times with DMF (6 ml each wash). Fmoc-protected glutamic acid methyl ester (Fmoc-Glu(O^{t}Me)-OH, 151 mg, 3 eq.), BOP (200 mg, 3 eq.), DIEA (238

μl, 6 eq.) and DMF (6 ml) were added to the resin and the mixture stirred for 30 min. This step was repeated. The resin was subsequently filtered and treated with Ac_2O/piperidine (5 ml) for 10 min. and washed three times with DMF (6 ml/wash). 30% piperidine/DMF (6 ml) was added to the resin and the resulting slurry was stirred for 20 min, to remove the Fmoc group. Fmoc-Gly (92 mg, 3 eq.) was coupled to the free amine of the bound glutamic acid methyl ester with BOP (137 mg, 3 eq.), DIEA (163 μl, 6 eq.) in DMF (6 ml.) The reaction was allowed to proceed for 30 min. This was repeated and the resin was filtered.

Acetic anhydride and piperidine (5 ml) were then added to cap any free amine groups on the resin. The resin was then washed with DMF (3 x ml) and the Fmoc groups were removed with 30% piperidine/DMF (5 ml, 20 min.) The resin was washed again with DMF (2 x 6 ml) and methanol (3 x 6 ml.) Cyclization was performed by refluxing the bound dipeptide in MeOH/triethylamine. Completeness of the reaction was determined by monitoring the amount of uncyclized dipeptide using the Kaiser ninhydrin test (cyclized product gives no reaction; uncyclized dipeptide gives a blue color). Following completion of the cyclization, crude cyclo-GlnGly was removed from the resin by reaction with 15 ml trifluoroacetic acid/H_2O (95/5) for one hour followed by filtration and removal of solvent. Crude yield: 70%. The crude product was purified by on a C-18 column with 0.1% TFA/H_2O as eluent. Yield of pure *cyclo*-GlnGly was 61%.



A: BOP reagent, DIEA, DMF (2x). B: Ac_2O, Pyridine. C: 30% Piperidine, DMF. D: Fmoc-Gly, BOP reagent, DIEA, DMF (2x). E: TEA, MeOH, reflux, F: TFA, H_2O.

Reaction Scheme IV

Example 2

Other Diketopiperazines

Using the method described in Example 1 and Reaction Scheme II, the following diketopiperazines were made (Table I):

Table I

DKP	Cyclization Time	Purification Method	Loading of 2nd Amino Acid (μmol/g)	% Yield†	% Yield‡
cyclo-AsnArg(Tos)	5 hr.	HPLC	116	70	31
cyclo-GlnGly	5 hr.	Column C18	162	61	38
cyclo-AsnHis(Bn)	14 hr.	HPLC	147	67	38
cyclo-GlnIle	4 days	HPLC	111	51	22
cyclo-GlnPro	14 hr.	HPLC	134	63	32
cyclo-Gln-p-Tyr	2 days	Column C-18	153	56	37
NH_2-Asn-*cyclo*-GlnTyr(PO_3H_2)	2 days	HPLC	154	68	40
cyclo-GlnTyr	2 days	HPLC	120	62	28

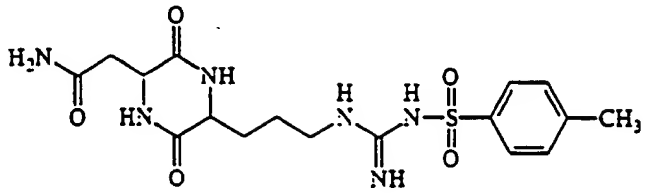
†: Yield based on loading of 2nd amino acid. ‡: Yield based on initial loading of the resin (260 μmol/g).

Example 3

Physical Data

The following data for each of the compounds in Table I was obtained.

Cyclo-AsnArg(Tos)

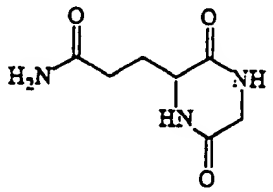


FAB-MS(M+H) :424

^{1}H NMR(300 MHz, D_2O) d 1.55 (m, 2H, -CH_2-), 1.75 (m, 2H, -CH_2-), 2.40 (s, 3H, -CH_3), 2.80 (d, J = 5.2 Hz, 2H, -CH_2-CO), 3.25 (m, 2H, -CH_2-N), 4.05 (br-s, 1H, -CH-N), 4.45 (t, J = 5.5 Hz, 1H, -CH-N), 7.41 (d, J = 7.7 Hz, 2H, aromatic), 7.80 (d, J = 8.2 Hz, 2H, aromatic)

^{13}C NMR(300 MHz, D_2O) d 29, 33, 38, 46, 49, 60, 62, 134, 138, 177, 178, 183.

Cyclo-GlnGly

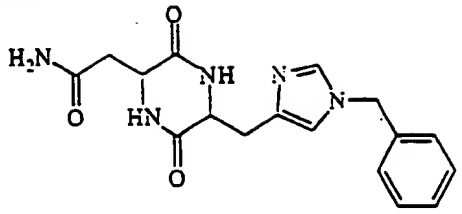


FAB-MS(M+H) :186

^{1}H NMR(300 MHz, D_2O) d 2.0 (m, 2H, -CH_2-), 2.25 (t, J = 7.9 Hz, 2H, -CH_2-CO-), 3.9 (q, J = 18.5 Hz, 2H, -CH_2-N), 4.05 (t, J = 5.3 Hz, 1H, -CH-N).

^{13}C NMR (300 MHz, D_2O) d 38, 38, 52, 60, 169, 179, 186.

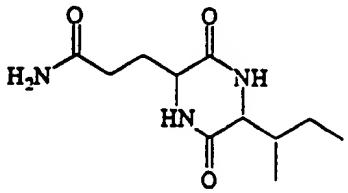
Cyclo-AsnHis(Bz)



ES-MS(M+H) :342

^{1}H NMR(300 MHz, D_2O) d 2.81 (d, J = 5.0 Hz, 2H, -CH_2-), 3.4 (m, -2H, -CH_2-CO-), 4.35 (t, J = 4.6 Hz, 1H, -CH-N), 4.44 (t, J = 4.8 Hz, 1H, -CH-N), 5.48 (s, 2H, -CH_2-N), 7.5 (s, 5H, aromatic), 7.58 (s, 1H, C=CH-), 8.99 (s, 2H, N=CH-).

Cyclo-GlnIle

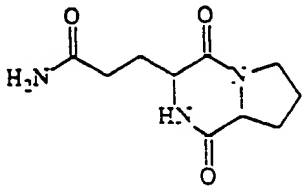


FAB-MS(M+H) :242

^{1}H NMR(300 MHz, D_2O) d 0.94 (m, 3H, -CH_3), 1.06 (m, 3H, -CH_3), 1.39 (m, 2H, -CH_2-), 2.04 (m, 1H, -CH-), 2.18 (m, 2H, -CH_2-), 2.46 (m, 2H, -CH_2-CO-), 4.21 (t, J = 7.1 Hz, 1H, -CH-N), 4.38 (t, J = 3.8 Hz, 1H, -CH-N)

^{13}C NMR (300 MHz, D_2O) d 19, 23, 32, 38, 39, 47, 62, 68, 178, 179, 186.

Cyclo-GlnPro

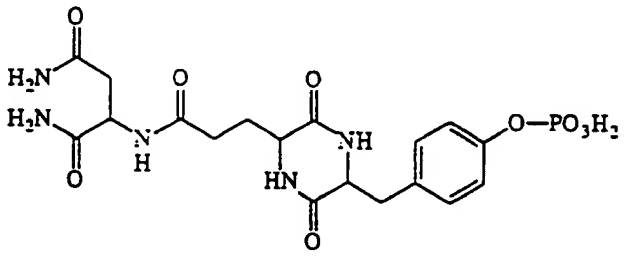


FAB-MS(M+H) :226

^{1}H NMR(300 MHz, D_2O) d 2.0(m, 2H, -CH_2-), 2.0 (m, 2H, -CH_2-), 2.2 (m, 2H, -CH_2-), 2.3 (br-d, J = 8.0 Hz, 2H, -CH_2-), 3.6 (m, 2H, -CH_2-CO-), 4.35 (m, 1H, -CH-N), 4.40 (m, 1H, -CH-N).

^{13}C NMR(300 MHz, D_2O) d 30, 33, 36, 38, 53, 63, 67, 175, 180.

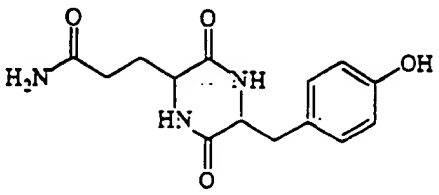
H_2N-Asn-*cyclo*-GlnTyr(PO_3H_2)



ES-MS(M-H) :484

^{1}H NMR(300 MHz, D_2O) d 1.35 (m, 2H, -CH_2-), 1.80 (m, 2H, -CH_2-), 2.80 (m, 2H, -CH_2-), 3.05 (m, 2H, -CH_2-CO-), 3.25 (m, 2H, -CH_2-CO-), 4.03 (m, 1H, -CH-N), 4.50 (br-s, 1H, -CH-N), 4.65 (m, 1H, -CH-N), 7.21 (br-s, 4H, aromatic)

Cyclo-GlnTyr



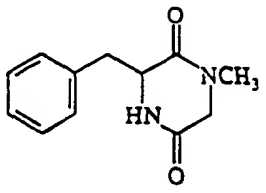
FAB-MS (M+H): 291.

^{1}H NMR (300 MHz, DMSO): d 1.12 (m, 1H, -CH-), 1.41 (m, 1H, -CH-), 1.78 (t, J = 2.5, 8.4 Hz, 2H, -CH_2-), 2.84 (dd, J = 4.9, 13.4 Hz, 1H, -CH-), 3.06 (dd, J = 4.3, 13.5 Hz, 1H, -CH_2-), 3.73 (tq, J = 1.4, 6.9 Hz, 1H, -NH-), 4.15 (tt, J = 1.5, 4.8 Hz, 1H, -CH-), 6.73 (dt, J = 2.8, 8.6 Hz, 2H, aromatic), 7.02 (dt, J = 2.0, 8.6 Hz, 2H, aromatic), 8.08 (d, J = 2.1 Hz, 1H, -NH-), 8.12 (d, J = 2.0 Hz, 1H, -NH-)

^{13}C NMR (300 MHz, DMSO): d 29, 39, 37, 53, 55, 115, 131, 156, 167, 167, 174.

Example 4

Synthesis of *Cyclo*-Phenylalanine-Sarcosine (*Cyclo*-Phe-Sar)



100 mg (220 mmol/g, 22 mmol) of Fmoc-protected phenylalanine (Phe-Fmoc), attached by an acetamide linker to TentaGel S resin (TentaGel S-Ac-Phe-Fmoc), was treated at 25°C for 10 minutes with a 25% solution of piperidine in DMF (1 mL). An aliquot of material was removed from the reaction mixture, and the optical density (OD) of the solution was measured for the fulvene-piperidine complex. The measured OD value of 1.369 indicated a resin loading of 176 mmol Fmoc-Phe/g of resin. The resin was rinsed with DMF (3 x 1 mL). Fmoc-Sarcosine (34 mg, 109 mmol), HATU (24 mg, 63 mmol), and DIEA (100 mL) were combined in DMF (to 1 mL) with the resin. The coupling reaction was allowed to proceed for 3 hours, at which time the solution was removed. The reaction was performed a second time. After the second coupling reaction, the resin was rinsed with DMF (3 x 1mL) and capped with Ac_2O/lutidine/THF (20

min). The resin was then washed with DMF (3 x 1 mL), treated with piperidine/DMF and measured as before to determine that the final loading of resin-bound dipeptide was 105 mmol/g. The resin was rinsed with DMF and EtOH. Finally, the resin was treated with 1% acetic acid (AcOH) in ^{i}PrOH (1 mL) at 60°C with monitoring by ninhydrin. The resin was filtered and the supernatant concentrated *in vacuo* to afford a white solid with a ^{1}H NMR spectrum consistent with the desired diketopiperazine (2.2 mg, 46%).

Example 5

Comparison of Acid and Base Catalyzed Cyclization of *Cyclo*-D-Gln-D-Tyr

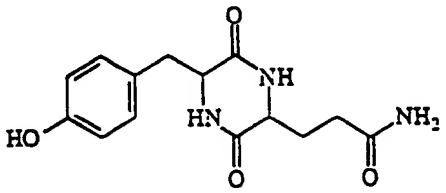
TentaGel S RAM Fmoc resin (240 mg, 250 mg/mol) was washed with DMF (2 x 7 ml). A solution of 30% piperidine in DMF (6 ml) was then added to the resin and the slurry was stirred for 20 minutes, before being washed with EtOH (2 x 6 ml) and DMF (6 ml). Fmoc-D-Glu(O^{t}Me)-OH (48 mg, 2 eq.), BOP (55 mg, 2 eq.), DIEA (44 ml, 4 eq.) in DMF solution (6 ml) were added to the resin, and the mixture was stirred for thirty minutes. This was repeated. After the second reaction, the resin was filtered and treated with acetic anhydride (Ac_2O) and pyridine (5 ml) for 10 minutes, after which the resin was washed with DMF (3 x 6 ml). A 30% solution of piperidine in DMF (6 ml) was then added to the resin and the mixture stirred for twenty minutes to release the Fmoc. The amount of Fmoc released was determined to be 147 mmol/g.

Fmoc-protected tyrosine (Fmoc-Tyr, 24 mg, 2 eq.) was coupled to the bound glutamine methyl ester using BOP (31 mg, 2eq.) and DIEA (25 ml, 4 eq) in DMF (6 ml). The reaction was allowed to proceed for thirty minutes, then repeated. After completion of the second reaction, the free amine groups of the resin were capped by the addition of Ac_2O/pyridine (5 ml). The resin was washed with DMF (3 x 6 ml) and the Fmoc groups removed by reaction of the protected, bound dipeptide with 30 % piperidine

in DMF (5 ml, 20 min.) The amount of Fmoc released from the resin was determined to be 131 mmol/g.

The resin was again washed with DMF (2 x 6 ml) and methanol (3 x 6 ml). Cyclization of the dipeptide to form the bound diketopiperazine was performed by refluxing the resin in MeOH/HOAc (99:1, 10 ml) for six hours. The progress of the reaction was monitored by the Kaiser ninhydrin test. Crude *cyclo*-D-Gln-D-Tyr was obtained by reaction with 5 ml of trifluoroacetic acid/water (95/5) for one hour, followed by filtration of the resin and removal of the solvent from the filtrate. The yield of crude product was 70%. Purification of the crude diketopiperazine on a prep-HPLC C-18 column using 0.1 % TFA in water. The yield of the purified diketopiperazine, based on the initial loading of the resin, was 45%. The yield based on the loading of the second amino acid was 77%.

cyclo-D-Gln-D-Tyr



Mass Spectrum (M+H): 291.

^{1}HNMR (300 MHz, DMSO) d 1.12 (m, 1H, -CH-), 1.41 (m, 1H, -CH-), 1.78 (t, J = 2.5, 8.4 Hz, 2H, -CH_2-), 2.84 (dd, J = 4.9, 13.4 Hz, 1H, -CH-), 3.06 (dd, J = 4.3, 13.5 Hz, 1H, -CH_2-), 3.73 (tq, J = 1.4, 6.9 Hz, 1H, -NH-), 4.15 (tt, J = 1.5, 4.8, 1H, -CH-), 6.73 (dt, J = 2.8, 8.6 Hz, 2H, aromatic), 8.08 (d, J = 2.1 Hz, 1H, -NH-), 8.12 (d, J = 2.0 Hz, 1H, -NH-).

^{13}CNMR (300 MHz, D_2O) d 31, 3, 39, 55, 116, 128, 133, 158, 168, 168, 175.

Additional cyclizations on other dipeptides were performed following the above-described protocol, but using both acid-mediated cyclization (1% HOAc in MeOH) and base-mediated cyclization (50% TEA in MeOH). The results are shown below in Table II. Yield was calculated using two methods. Yield determined by method a is based on the amount of amino acid loaded on the resin initially (260 mmol/g). Yield determined by method b is based on the loading of the second amino acid as determined by the amount of Fmoc released upon deprotection. Reaction time is measured in hours (hr.)

Table II

Acid- vs. Base-Mediated Cyclization of Diketopiperazines

	Acid-Mediated Yield: a/b/reaction time	Base-Mediated Yield: a/b/reaction time
cyclo-L-Gln-D-Tyr	37%/68%/6 hr.	27%/44%/24 hr.
cyclo-D-Gln-D-Tyr	45%/77%/6 hr.	24%/39%/24 hr.
cyclo-D-Gln-L-Tyr	38%/60%/6 hr.	23%/37%/24 hr.
cyclo-D-Gln-L-Tyr	40%/70%/overnight	22%/51%/4 days

Example 6

Preparation of N-Linked 1,4-Diketopiperazines

The following procedure can be used to form diketopiperazines in a cleavable format through the use of the Knorr linker, either on TentaGel or Bachem resin, or in a non-cleavable format on TentaGel S NH_2 resin or Pharmacia Mono A resin.

p-[(R,S)-a-[1-(9H-Fluoren-9-yl)-methoxyformamido}-2,4-dimethoxybenzyl]-phenoxyacetic acid (590mg, 1.1 mmol), HBTU (760 mg, 2.0 mmol), and DIEA (1 mL) were combined at room temperature with TentaGel S NH_2 resin (1g, 0.23 mmol) in 3:1 DMF:CH_2Cl_2 (30 mL). The reaction slurry was mixed while N_2 was bubbled through the solution for 30 minutes, at which time the liquid phase removed by vacuum filtration. The beads were rinsed with DMF (2 x 30 mL) and treated with 25% piperidine in DMF (10 mL) for 20 minutes. An aliquot of the solution phase was removed, diluted, and measured at 302 nm for the OD of the fulvene-piperidine complex. Analysis of this mixture indicated a loading value of 135 mmol/g of resin. The beads were rinsed with DMF (5-10 x 10 mL until no piperidine smell is detected). TentaGel resin having the Knorr-Fmoc linker attached thereon is also available commercially.

Bromoacetic acid (160 mg, 1.2 mmol) and 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride (WSC-HCl) (470 mg, 2.4 mmol) were combined with the above-described TentaGel resin in 3:1 DMF:CH_2Cl_2 at room temperature. The slurry was mixed by bubbling N_2 into the solution for 20 minutes, after which the solution phase was removed by vacuum filtration. The beads were rinsed with DMF (3 x 10 mL) and THF (10 mL). The free amino groups were capped by treatment with 4 mL each of solutions of Ac_2O, pyridine and imidazole, THF. The slurry was mixed by bubbling for 20 minutes. The beads were then filtered by vacuum filtration and rinsed with THF (3 x 10 mL) and DMF (10 mL).

A 100 mg portion of the dried solid supported bromoacetamide was treated at 60°C for two hours with a solution (1 mL) of glycine methyl ester hydrochloride (154 mg, 1.2 mmol), *N*,*N*-dimethylaminopyridine (10 mg), and diisopropylethylamine (100 mL) in DMSO, as described by Zuckermann, *et al.*[26] The reaction slurry was subsequently cooled to room temperature and rinsed with DMF (3 x 1 mL). Occasionally, the resin will float in the DMSO solution. In such cases, a small amount of

DMF is added and the slurry is vortexed briefly and centrifuged. The beads subsequently pelleted.

The resin-bound glycine-bromoacetamide adduct was acylated with Fmoc-glycine-NCA (32 mg, 0.1 mmol) and DIEA (100 mL) in toluene (to 1 mL) at room temperature for 11-48 hours. Longer reaction times will increase the coupling yield slightly. The resin was then pelleted by centrifugation and rinsed with toluene (3 x 1 mL) and DMF (1 mL). The Fmoc group was cleaved by treating the resin for 20 minutes with 25% piperidine in DMF (1 mL). The optical density of a 10 mL aliquot of the cleavage mixture diluted with 990 mL of DMF was measured at 302 nm, and the amount of loading of the glycine acylation product per gram of resin was determined. Typical loading values ranged from 90-120 mmol/g.

The resin bound dipeptide was thoroughly rinsed with DMF (5-10 x 1 mL) until no piperidine smell remained and rinsed again with EtOH (1 mL). The resin was then treated at 60°C with triethylamine (200 mL) in EtOH (500 mL). The progress of cyclization was followed by ninhydrin staining of small aliquots of resin which had been removed and rinsed with EtOH. Cyclization typically required overnight (> 10 h) to reach completion.

Additional amino acids were incorporated onto the resin prior to DKP formation as illustrated below.

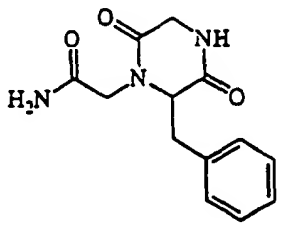
Fmoc-Asn(Trt) was double-coupled with HBTU and DIEA in DMF to TentaGel S NH_2 (230 mmol/g) resin (1 g), to afford after capping with Ac_2O and Fmoc cleavage with piperidine/DMF the amino acid supported on beads at a loading of 142 mmol/g. Bromoacetic acid was coupled with EDAC in DMF onto the amine terminus of the resin-bound material and capped with Ac_2O. A 100 mg sample of the beads was treated with Tyr-OMe-HCl (1.25 M) with DIEA and DMAP (cat.) in DMSO at 80°C for 14 hours. The rinsed beads were then treated with Fmoc-Gly-NCA and DIEA in toluene at room temperature for 48 h to afford after Fmoc release resin with a loading of the tripeptide of approximately 131 mmol/g. The

bead sample was treated with triethylamine (200 mL) (TEA) in EtOH (500 mL) at 80°C and was monitored by ninhydrin staining of small aliquots of resin. Completion of the cyclization step was indicated after 4 days by a negative ninhydrin test. The resin was subsequently rinsed with EtOH and dried under vacuum.

A similar procedure using Gly-OMe-HCl followed by acylation with Fmoc-Gly-NCA (14 h coupling) on a 55 mg sample of resin afforded an loading number of 135 mmol/g. In the case of the less hindered amino acids, cyclization appears faster with a negative ninhydrin test occurring after typically in 5-14 hours.

Example 7

Synthesis of Acetamide-*cyclo*-L-Phe-L-Gly

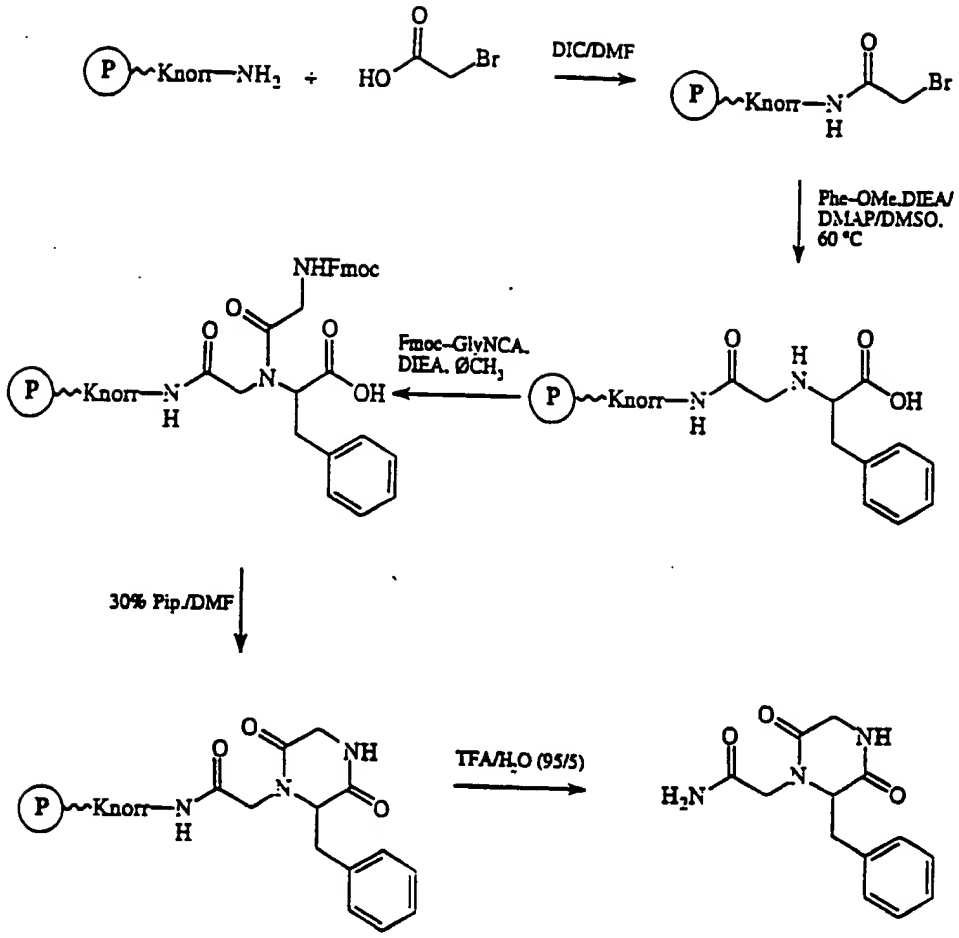


Referring to Reaction Scheme V below, TentaGel S RAM Fmoc resin (310 mg, 250 μmol/g) was washed with DMF (2x7 ml), and a 30% piperidine/DMF solution (2 ml) was added to the resin. The slurry was allowed to stir for 20 min. The resin was then washed with EtOH (2x6 ml) and DMF (3x6 ml). Bromoacetic acid (50 mg, 3 eq.), diisopropylcarbodiimine (50 μl, 6 eq.) and DMF were added to the resin and this mixture was stirred for 30 min. This was repeated, and the resin was filtered and treated with an acetic anhydride/pyridine solution (2 ml) for 5 min. after which the resin was washed with DMF (2x2 ml) and DMSO (2x2 ml). Phenylalanine methyl ester (Phe-OMe, 2.5 M in DMSO) 2 ml, DTEA (399 μl), and DMAP were added to the resin and the mixture was stirred overnight at 60°C. The resin was filtered and washed with MeOH (2 x 2

ml) and toluene (3 x 2 ml). Fmoc-L-Gly-NCA (100 mg, 6 eq) was coupled to the resin with DIEA (100 μl) in toluene (2 ml) overnight at room temperature. The resin was washed with DMF (3x2 ml), and the Fmoc group was removed using a 30% piperidine/DMF solution (2 ml, 20 min.) The Fmoc released from the resin was determined to be 96 μmol/g. The resin was rinsed twice with methanol and Kaiser's test was performed which showed the cyclization to be complete. Crude acetamide-Cyclo-L-Phe-L-Gly was obtained by treatment of the resin with 3 ml of a TFA/H_2O solution (95/5) for an hour, after which the resin was filtered and the solvent removed from the filtrate. Crude yield: 50%. The crude DKP was purified using a prep-HPLC C-18 column with 0.1% TFA in H_2O as eluent. The amount of material recovered was 2 mg. The yield based on the initial loading of the resin was 10%. The yield calculated from the loading of the 2nd amino acid was 26%.

Mass spectrum (M+H): 261

^{1}H NMR (300 MHz, D_20) d: 2.36 (d, J = 18.3 Hz, 1H, -CH-), 3.3 (t, 2H, -CH2-), 3.55 (d, J = 18.3 Hz, 1H, -CH-), 4.00 (d, J = 16.8 Hz, 1H, -CH-), 4.34 (t, J = 4.51, 3.98 Hz, 1H, -CH-), 4.60 (d, J = 16.7 Hz, 1H, -CH-), 7.21 (m, 2H, aromatic), 7.42 (m, 2H, aromatic). ^{13}C NMR (300 MHz, D_20) d: 45, 51, 55, 70, 136, 137, 138, 142, 176, 180, 194.



Reaction Scheme V

Example 8

Formation of Amino Acid Fluorides *In Situ*

Amino acid fluorides are versatile reagents in peptide synthesis.[27] A procedure is described for producing such derivatives *in situ*.

A solution of cyanuric fluoride (40 mL, 0.47 mmol) and pyridine (40 mL) was prepared in CH_2Cl_2 (1 mL) and mixed at room temperature for 7 hours. Fmoc-Alanine (62 mg, 0.2 mmol) was added to the mixture

and the reaction was allowed to proceed for 30 minutes. During the course of this reaction and yellowish white precipitate forms. The reaction mixture was centrifuged and the yellow supernatant added to a sample of TentaGel S NH_2 resin (100 mg, 23 mmol). The reaction was allowed to proceed for approximately 15 hours. After rinsing with EtOH, H_2O, DIEA/THF and DMF, the Fmoc was cleaved by 25% piperidine/DMF and the loading of amino acid on the resin was determined to be 86 mmol/g.

A double coupling (20 minute couplings) procedure to attach Fmoc-Gly to the above rinsed resin using HBTU gave a loading (after Fmoc-cleavage) of 87 mmol/g.

Example 9

Preparation of N-Alkylated Diketopiperazines

A. Reductive Aminations

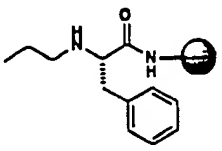
After deprotection of Fmoc•Phe-Tentagel PHB resin (220 mg, 0.103 mmol) with 30% piperidine/DMF, the resin was washed with DMF and ether, then dried. The deprotected resin was washed with trimethylorthoformate (5 ml) and then suspended in trimethylorthoformate (3 ml). To the suspension was added the aldehyde (1.03 mmol, 10 equiv). After 30 minutes, a solution of $NaCNBH_3$ (1.03 mmol, 10 equiv) in trimethylorthoformate (4 ml) was added. Depending on the reactivity of the aldehyde, as discussed above, a solution of 1% methanol or 1% acetic acid in trimethylorthoformate is added after 5 minutes. The resin was then washed with methanol, ether, then dried under vacuum. The resin is initially blue when subject to a ninhydrin test, secondary amines are pink, and tertiary amines are colorless.

Depending on the resin that is used, the N-alkylated-Phe may be cleaved as the free acid by treatment with 95% trifluoroacetic acid/water. After concentration under vacuum, MS analysis may be performed to determine if any over alkylation has occurred.

Using this procedure the following N-alkylated-phenylalanine derivatives have been prepared without any evidence of over-alkylated material: N-propyl-phenylalanine; N-isobutyryl-phenylalanine; N-benzyl-phenylalanine.

B. Secondary Acylations

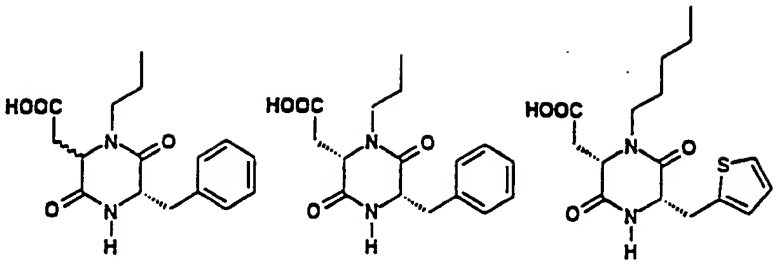
All resin and Fmoc amino acids were dried under vacuum in the presence of P_2O_5 overnight prior to use. N-propyl-Phe-Tentagel resin (25 mg, 237 mmol/g) is treated with solvent (dichloromethane, or NMP), Fmoc•amino acid (0.1 mmol), DIEA (0.3 mmol), and coupling reagent (0.1 mmol) added. After 24-hours the resin was washed with NMP and ether, then dried under vacuum. The Fmoc group was removed in 30% piperidine/NMP for 30-minutes and the dibenzofulvene-piperidine adduct spectrophotometrically measure at 302 nm to determine coupling yields.

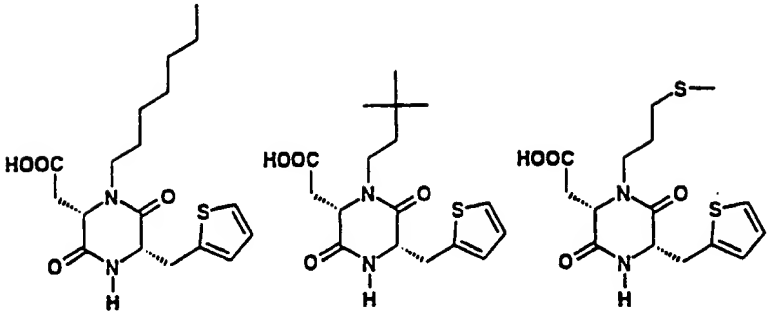


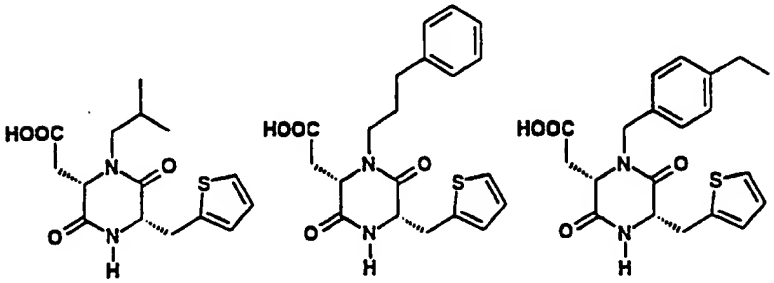
amino acid	coupling reagent	solvent	yield
Gly	BoPCl PyBrOP HATU	NMP	45% n/a 41%
Gly	BoPCl PyBrOP HATU	DCM	63% 66% 96%
Phe	HATU	DCM	54%
Val	BoPCl PyBrOP HATU	DCM	4% 3% 13%

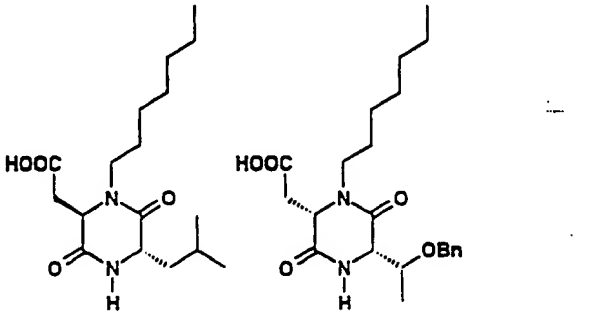
• room temperature; 100 mM amino acid; 100 mM coupling reagent; 300 mM DIEA.
• yield determined by A_{302} measurements of dibenzofulvene/piperidine adduct.

Using the above procedures, the following compounds were prepared:









Thus, the present invention is seen to provide libraries of diverse diketopiperazines bound to a support and methods for synthesizing diketopiperazines on a solid support. The libraries of the present invention have utility in the area of drug design as they can be screened against biological substances to identify compounds which have desirable biological activity. The libraries of the invention are also well adapted to structure-function studies of activity, especially quantitative structure activity relationships. Using the libraries of the invention, optimal lead compounds may be identified for additional study.

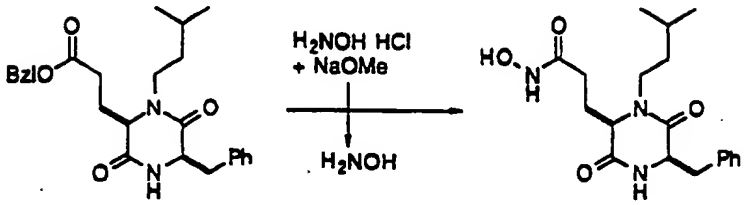
Example 10

Preparation of Hydroxamic Acids

To a solution of the carboxylic acid (1 eq.), EDC (1.5 eq.) and DIEA (3-4 eq.) in a nonpolar solvent, such as dichloromethane, is added an O-protected hydroxylamine (about 1.5 eq.). If the acid is not soluble in dichloromethane, DMF may be added. The reaction is performed under an inert atmosphere and can be monitored by TLC. Reaction times vary from 1-6 hours.

Example 11

Alternative Synthesis of Hydroxamates



139 mg (2 mmol) hydroxylamine hydrochloride were place under argon in a dry flask and 0.5 M NaOMe solution in MeOH (6 mL, 3 mmol) was added. The cloudy solution was stirred vigorously for 10 minutes at room temperature. The NaCl-precipitation was then allowed to settle down and the clear supernatant taken up in a syringe. The solution (1 mL, 0.33 mmol) was added through a HPLC-syringe filter to the benzylester-DKP (6.7 mg, 0.015 mmol) under argon. It was allowed to react for about 15 minutes and then concentrated. The white residue was re-dissolved in acetonitrile/water 1:1 and AG 50W-X8 cation exchange resin (Biorad) added until pH 2 was reached. The resin was filtered off and the solution concentrated. Monitor by TLC with $FeCl_3$/EtOH/1N HCl.

Further to Example 9, the following examples illustrate methods for achieving reductive alkylation of a primary amine with a large excess of a carbonyl component and sodium cyanoborohydride without the concomitant formation of tertiary amines common with the prior art when using large excesses of the carbonyl component and reducing agent. These examples are applicable to both solution phase and solid phase syntheses. In some embodiments, the reductive alkylation is accomplished by allowing imine formation to occur followed (approximately 30 minutes later) by reduction optionally in the presence of a proton source (e.g., 1 volume percent of MeOH or HOAc).

Example 12

N-propyl-L-phenylaninamide-TsOH

To phenylalaninamide (0.250 g, 1.52 mmol) dissolved in trimethylorthoformate (20 mL) under argon, was added propionaldehyde (1.1 mL, 15.2 mmol) followed by $NaCNBH_3$ (0.478 g, 7.61 mmol) dissolved in trimethylorthoformate (20 mL). After 1 hour, the reaction mixture was cooled to 0°C, quenched with 2% aqueous HCl (50 mL), washed with ether (25 mL x 3). The ether phase was extracted with 2% aqueous HCl (2 x 25 mL), the aqueous phases were combined, cooled to 0°C, treated with concentrated NaOH until basic, then extracted with ether (4 x 25 mL). The ether phase was dried over $MgSO_4$ and filtered. The filtrate was treated with p-toluenesulfonic acid (1.672 mmol) dissolved in ether (10 mL) then placed in the freezer. The precipitate was collected by vacuum filtration, washed with ether, and dried under vacuum to provide the title compound as a colorless solid (0.441 g, 77%).

Example 13

N-isobutyl-L-Phenylalaninamide-TsOH

To phenylalaninamide (0.255 g, 1.55 mmol) dissolved in trimethylorthoformate (20 mL) under argon, was added isobutyraldehyde (1.4 mL, 15.4 mmol) followed by $NaCNBH_3$ (0.487 g, 7.75 mmol) dissolved in trimethylorthoformate (20 mL). After 1 hour, the reaction mixture was worked up as described in Example 12 to provide for the title compound as a colorless solid (0.420 g, 69%).

Example 14

N-(2,2-dimethylpropyl)-L-Phenylalaninamide-TsOH

To phenylalaninamide (0.125 g, .761 μmmol) dissolved in trimethylorthoformate (10 mL) under argon, was added trimethylacetaldehyde (0.83 mL, 7.64 mmol) followed by $NaCNBH_3$ (0.245 g, 3.89 mmol) dissolved in trimethylorthoformate (10 mL). After 1 hour,

the reaction mixture was worked up as described in Example 12 to provide for the title compound as a colorless solid (0.249 g, 80%).

Example 15

N-benzyl-L-Phenylalaninamide-TsOH

To phenylalaninamide (0.259 g, 1.58 mmol) dissolved in trimethylorthoformate (20 mL) under argon, was added acetic acid (0.4 mL) and benzaldehyde (1.6 mL, 15.7 mmol). After 30 minutes, $NaCNBH_3$ dissolved in trimethylorthoformate (20 mL) was added. After 1 hour, the reaction mixture was worked up as described in Example 12 to provide for the title compound as a colorless solid (0.501 g, 74%).

Example 16

N-isopropyl-L-Phenylalaninamide-TsOH

To phenylalaninamide (0.252 g, 1.53 mmol) dissolved in trimethylorthoformate (20 mL) under argon, was added acetic acid (0.4 mL) followed by acetone (1.1 mL, 15.0 mmol). After 30 minutes, $NaCNBH_3$ (0.482 g, 7.67 mmol) dissolved in trimethylorthoformate (20 mL) was added. After 1 hour, the reaction mixture was worked up as described in Example 12 to provide for the title compound as a colorless solid (0.477 g, 82%).

Example 17

N-cyclohexyl-L-Phenylalaninamide-TsOH

To phenylalaninamide (0.257 g, 1.56 mmol) dissolved in trimethylorthoformate (20 mL) under argon, was added acetic acid (0.4 mL) and cyclohexanone (1.6 mL, 15.4 mmol). After 30 minutes, $NaCNBH_3$ (0.492 g, 7.83 mmol) dissolved in trimethylorthoformate (20 mL) was added. After 1 hour, the reaction mixture was worked up as described in Example 12 to provide for the title compound as a colorless solid (0.540 g, 83%).

Example 18

N-(2,2-dimethylpropyl)-L-Valinamide-TsOH

To valinamide (0.120 g, 1.03 mmol) dissolved in trimethylorthoformate (15 mL) under argon, was added trimethylacetaldehyde (1.1 mL, 10.1 mmol) followed by $NaCNBH_3$ (0.324 g, 5.16 mmol) dissolved in trimethylorthoformate (15 mL). After 1 hour, the reaction mixture was worked up as described in Example 12 to provide for the title compound as a colorless solid (0.304 g, 82%).

Example 19

N-propyl-Phe-Phe-amide-TFA

To NH_2-Phe-Phe on PAL resin (170 mg, 0.046 mmol) suspended in trimethylorthoformate (2 mL) under argon, was added propionaldehyde (74 μL, 1.03 mmol) and the system was then shaken for 30 minutes. $NaCNBH_3$ (64 mg, 1.03 mmol) dissolved in trimethylorthoformate (1 mL) was added. After 10 minutes, the resin was drained, washed with methanol and ether and dried. The N-alkylated peptide was cleaved from the resin with 95% trifluoroacetic acid/water (3 mL) for 3 hours, filtered and the rsin washed with trifluoroacetic acid. After concentration of the filtrate, the crude material was purified by preparative HPLC using a water/acetonitrile/trifluoroacetic acid gradient to yield the title compound as a colorless solid (13 mg, 61%).

Example 20

N-(2-methylpropyl)-L-Phe-Phe-amide-TFA

To NH_2-Phe-Phe on PAL resin (190 mg, 0.051 mmol) suspended in trimethylorthoformate (2 mL) under argon, was added isobutyraldehyde (94 μL, 1.03 mmol) and the system was then shaken for 30 minutes. $NaCNBH_3$ (64 mg, 1.03 mmol) dissolved in trimethylorthoformate (1 mL) was added. After 10 minutes, the reaction was worked up as described

above in Example 19 to provide the title compound as a colorless solid (21 mg, 84%).

Example 21

N-benzyl-Phe-Phe-amide-TFA

To NH_2-Phe-Phe on PAL resin (226 mg, 0.063 mmol) suspended in trimethylorthoformate (2 mL) under argon, was added benzaldehyde (65 μL, 0.63 mmol) and the system was then shaken for 30 minutes. $NaCNBH_3$ (40 mg, 0.63 mmol) dissolved in trimethylorthoformate (1 mL) was added followed by acetic acid (30 μL). After 10 minutes, the reaction was worked up as described above in Example 19 to provide the title compound as a colorless solid (24 mg, 75%).

Example 22

N-(2,2-dimethylpropyl-Val-Phe-amide-TFA

To NH_2-Val-Phe on PAL resin (304 mg, 0.09 mmol) suspended in trimethylorthoformate (4.5 mL) under argon, was added 2,2-dimethyl-propionaldehyde (98 μL, 0.9 mmol) and the system was then shaken for 30 minutes. $NaCNBH_3$ (57 mg, 0.9 mmol) dissolved in trimethylorthoformate (1.5 mL) was added followed by acetic acid (50 μL). After 10 minutes, the reaction was worked up as described above in Example 19 to provide the title compound as a colorless solid (29 mg, 72%).

Other diketopiperazines prepared include those set forth in Figure 6 (R^2, R^3 = alkyl, aryl, heteroaryl, alkylaryl, alkylheteroaryl, substituted alkyl, substituted aryl, substituted heteroaryl, and the like) which illustrates the formation using cysteine as the first amino acid attached to a PAM-OH resin. In this figure, the Fmoc-N-protected cysteine is coupled to the resin via the carboxyl group to provide the resin bound derivative thereof. Conventional removal of the Fmoc group followed by reductive amination and subsequent coupling of the second amino acid and cyclization provides for the diketopiperazines. It is understood, of course, that the PAM-OH

resin and the Fmoc groups are for illustrative purposes only and that other groups could be used in place thereof. Additionally, on either TentaGel SAM-resin or the illustrated PAM resin, acidic cyclization provided for one predominate isomer (i.e., no racemization with TentaGel SAM-resin but some with PAM (1:4 ratio of isomers) whereas basic cyclization (i.e., 1% triethylamine, toluene) proceeded faster but with more racemization).

While the invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that the foregoing and other changes in the form and details may be made therein without departing from the spirit or scope of the invention.

References

The following references are cited in this application as superscript numbers and are incorporated herein by reference.

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2. Srinivasachari et al., "Piperazine-2,5-diones and Related Lactim Ethers" in ADVANCES IN HETERCYCLIC CHEMISTRY, Vol. 57, pp. 187-260 (Academic Press 1993).
3. *Id.*
4. *Id.*
5. See, e.g., Giron-Forest et al., "Bromocryptine Methanesulphonate" in ANALYTICAL PROFILES OF DRUG SUBSTANCES, Vol. 8 (Academic Press 1979).
6. See, e.g., Williams et al., *Chem. Rev.* 88:511-540 (1988).
7. See, Sammes, *supra*, page 87.
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9. See, e.g., U.S. Patent No. 3,752,888 to Fluckiger et al.
10. See, e.g., Hwang et al., *Biochemistry*, 88:4756-4763 (1983); Shen et al., *Proc. Nat. Acad. Sci USA*, 82:673-676 (1985; Shimazaki et al.,

Chem. Pharm. Bull. 35(8):3527-3530 (1987); and Norihiko et al., *J. Med. Chem.* 30:1706-1709 (1987).

11. See, e.g., Shiosaki et al., "Towards development of peptidomimetics: Diketopiperazine templates for the Trp-Met segment of CCK-4."
12. See, e.g., Kucharczyk et al., *J. Med. Chem.*, 36:1654-1661 (1993).
13. Williams, R., SYNTHESIS OF OPTICALLY ACTIVE α-AMINO ACIDS (Pergammon, 1989).
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15. SYNTHETIC PEPTIDES: A USER'S GUIDE, Grant, Ed. (Freeman, 1992).
16. SOLID PHASE PEPTIDE SYNTHESIS; A PRACTICAL APPROACH, Atherton et al., Eds. (IRL Press 1989).
17. See, e.g., Grant, *supra*.
18. Palom et al., *Tetrahedron Lett.*, 34:22195-2198.
19. *Peptide Research*, 4:19 (1991).
20. Ho et al., *Peptide resarch*, 6:10-12 (1993) and Coy et al., *Tetrahedron*, 44:835-841 (1988).
21. See, e.g., Srinivasachari et al., *supra*.
22. See, e.g., Sammes, *supra*.
23. See, PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS (Innis, M., Gelfand, D., Sninsky, J., and White, T., Academic Press, San diego, 1990); See also U.S. Patent Nos. 4,683,22 and 4,965,188.
24. See, e.g., *J. Immune Methods*, 102:259-274 (1987).
25. See, U.S. Patent No. 5,288,514 to Ellman.
26. *J. Am. Chem. Soc.*, 114:10646 (1992).
27. See, e.g., Carpino et al., *J. Am. Chem. Soc.*, 112:9652 (1990); and Bertho et al., *Tetrahedron Lett.*, 32:1303.

WHAT IS CLAIMED IS:

1. A library of diverse diketopiperazine structures, comprising a plurality of solid supports having a plurality of surface-bound diketopiperazines, wherein the diketopiperazines bound to each of the solid supports are substantially homogeneous and have a composition different from diketopiperazines bound to selected other solid supports.

2. The library of Claim 1, wherein each of the solid supports further comprises a linker which linker is either a cleavable or a non-cleavable linker.

3. The library of Claim 1, wherein each of the solid supports further comprises a surface-bound tag.

4. A compound comprising a diketopiperazine covalently bound to a solid support optionally through a linker wherein the diketopiperazine is bound first to a linker and the linker is then bound to the solid support.

5. The compound of Claim 4, wherein a carbon atom of the diketopiperazine is bound to the linker.

6. The compound of Claim 4, wherein a nitrogen atom of the diketopiperazine is bound to the linker.

7. A method of synthesizing diketopiperazines on a solid support, comprising the steps of:

(a) on the surface of a solid support, providing a bound first amino acid derivative; and

b) exposing the supports to conditions effective to form a diketopiperazine.

8. The method of Claim 7, wherein the step of providing a bound first amino acid derivative comprises the step f: binding a first amino acid to the support to form a bound first amino acid derivative.

9. The method of Claim 7, wherein the step of exposing the supports to conditions effective to form a diketopiperazine comprises the steps of:

(i) reacting the bound first amino acid derivative with a second amino acid derivative under conditions effective to form a bound dipeptide derivative; and

(ii) reacting the bound dipeptide derivative under conditions effective to cyclize the bound dipeptide derivative and form a diketopiperazine.

10. A method for preparing a synthetic compound library produced by synthesizing on each of a plurality of solid supports a single compound wherein each compound comprises at least one diketopiperzine group, which library is synthesized in a process comprising:

a) providing a plurality of supports, each support further comprising a bound first amino acid derivative;

b) reacting the supports with a second amino acid derivative under conditions effective to produce a bound dipeptide derivative; and

c) reacting the bound dipeptide derivative under conditions effective to cyclize the bound dipeptide derivative to form a diketopiperazine, provided that at least one of the following conditions is met:

(i) at least two different first bound amino acid derivative are used; or

(ii) at least two different second amino acid derivatives are used.

11. The method according to Claim 10 wherein each of the supports contains a different compound.

12. A method of synthesizing a N-alkylated diketopiperazine, comprising the steps of:

(a) providing a first amino acid derivative bound to the solid support;

(b) reductively aminating the support-bound first amino acid derivatives to form a first mono-alkvlated amino acid derivative;

(c) reacting the first mono-alkylated amino acid derivative with a second amino acid derivative under conditions effective to form a pep tide bond wherein an N-alkylated dipeptide is formed; and

(d) reacting the N-alkylated dipeptide under conditions effective to cyclize the N-alkylated dipeptide and release the resulting N-alkylated diketopiperazine from the solid support.

13. A method of synthesizing a library of N-alkylated diketopiperazine, comprising the steps of:

(a) providing a first amino acid derivative bound to the solid support;

(b) reductively aminating the support-bound first amino acid derivatives to form a first mono-alkylated amino acid derivative;

(c) reacting the first mono-alkylated amino acid derivative with a second amino acid derivative under conditions effective to form a pep tide bond wherein an N-alkylated dipeptide is formed; and

(d) reacting the N-alkylated dipeptide under conditions effective to cyclize the N-alkylated dipeptide and release the resulting N-alkylated diketopiperazine from the solid support, provided that at least two different N-alkylated diketopiperazines are formed.

14. The method of Claim 13 wherein the step of providing a first amino acid derivative bound to the solid support further comprises the step of: providing a plurality of supports, each support further comprising a bound first amino acid derivative.

15. The method of Claim 10 or 13 wherein the step of providing a first amino acid derivative bound to the solid support further comprises the step of:

(i) providing a plurality of reaction vessels, each vessel containing a plurality of solid supports, each support comprising a plurality of bound first amino acid derivatives, wherein the first amino acid derivatives bound to each of the solid supports are substantially homogeneous and have a composition different from first amino acid derivatives bound to selected other solid supports.

16. The method according to Claim 10 or 13, wherein the step of providing a first amino acid derivative bound to the solid support further comprises the steps of:

(i) providing a plurality of reaction vessels, each vessel containing a plurality of solid supports, each support comprising a plurality of bound first amino acid derivatives, wherein the first amino acid derivatives bound to each of the solid supports are substantially homogeneous and have a composition different from first amino acid derivatives bound to selected other solid supports;

(ii) pooling the supports; and

(iii) optionally apportioning the supports in a second plurality of reaction vessels.

17. The method according to Claim 13, wherein the step of reductively aminating the support-bound first amino acid derivatives to form first mono-alkylated amino acid derivatives comprises the step of:

(i) treating the bound first amino acid derivative with an aldehyde and a reducing agent in the presence of a dehydrating agent.

18. The method of Claim 17, wherein the reducing agent is sodium cyanoborohydrate and the dehydrating agent is trimethylorthoformate.

19. The method of Claim 13, further comprising the step of: prior to the step of reductively aminating the bound first amino acid derivative, apportioning the beads among a plurality of reaction vessels.

20. The method of Claim 19, wherein the step of reductively aminating the support-bound first amino acid derivatives to form a first mono-alkylated amino acid derivative further comprises the steps of:

(i) providing a plurality of reaction vessels, each vessel containing a plurality of support-bound first amino acid derivatives, wherein the first amino acid derivatives bound to each of the solid supports are substantially homogeneous and have a composition different from first amino acid derivatives bound to selected other solid supports;

(ii) optionally pooling the supports; and

(iii) treating the bound first amino acid derivatives with an aldehyde and a reducing agent in the presence of a dehydrating agent.

21. The method of Claim 19, wherein the step of reductively aminating the support-bound first amino acid derivatives to form a first mono-alkylated amino acid derivative further comprises the steps of:

(i) providing a first plurality of reaction vessels, each vessel containing a plurality of support-bound first amino acid derivatives, wherein the first amino acid derivatives bound to each of the solid supports are substantially homogeneous and have a composition different from first amino acid derivatives bound to selected other solid supports;

(ii) pooling the supports;

(iii) apportioning the supports in a second plurality of reaction vessels; and

(iv) treating the bound first amino acid derivative with an aldehyde and a reducing agent in the presence of a dehydrating agent.

22. The method of Claim 21, further comprising the step of pooling the beads.

23. The method of Claim 13, further comprising the step of:

prior to the step of reacting the first mono-alkylated amino acid derivative with a second amino acid derivative under conditions effective to form a pep tide bond wherein an N-alkylated dipeptide is formed, apportioning the beads among a plurality of reaction vessels.

24. The method of Claim 23, wherein the step reacting the first monoalkylated amino acid derivative with a second amino acid derivative under conditions effective to form a peptide bond wherein an N-alkylated dipeptide is formed; an further comprises the steps of:

(i) providing a plurality of reaction vessels, each vessel containing a plurality of support-bound first mono-alkylated amino acid derivatives, wherein the first mono-alkylated amino acid derivativess bound to each of the solid supports are substantially homogeneous and have a composition different from first mono-alkylated amino acid derivatives bound to selected other solid supports; and

(iv) reacting the first mono-alkylated amino acid derivatives with a second amino acid derivative under conditions effective to form a pep tide bond wherein an N-alkylated dipeptide is formed.

25. The method of Claim 24 wherein a different second amino acid derivative is present in each of the reaction vessels.

26. The method of Claim 23, wherein the step reacting the first mono-alkylated amino acid derivative with a second amino acid derivative

under conditi ns effective to form a pep tide bond wherein an N-alkylated dipeptide is formed; an further comprises the steps f:

(i) providing a plurality of reaction vessels, each vessel containing a plurality of support-bound first mono-alkylated amino acid derivatives, wherein the first mono-alkylated amino acid derivativess bound to each of the solid supports are substantially homogeneous and have a composition different from first mono-alkylated amino acid derivatives bound to selected other solid supports;

(ii) pooling the supports;

(iii) optionally apportioning the supports in a second plurality of reaction vessels; and

(iv) reacting the first mono-alkylated amino acid derivatives with a second amino acid derivative under conditions effective to form a pep tide bond wherein an N-alkylated dipeptide is formed.

27. The method of Claim 26, further comprising the step of pooling the beads.

28. The method of Claim 26 wherein a different second amino acid derivative is present in each of the reaction vessels.

29. A method of screening diketopiperazine derivatives for biological activity, comprising the steps of:

(a) forming a library of support-bound diketopiperazine derivatives;

(b) exposing the diketopiperazines to a biologically active substance; and

(c) determining whether any of the diketopiperazines binds to the biologically active substance.

30. The method of Claim 29, wherein the biologically active substance is a protein.

31. The method of Claim 29, wherein the library comprises a plurality of polymer beads having diketopiperazines bound thereon, wherein the diketopiperazines bound to each of the beads are substantially homogeneous and have a composition different from diketopiperazines bound to selected other beads.

FIG. 1A

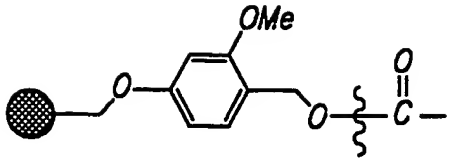


FIG. 1B

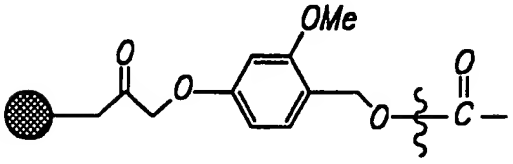


FIG. 1C

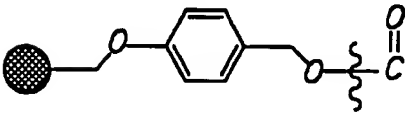
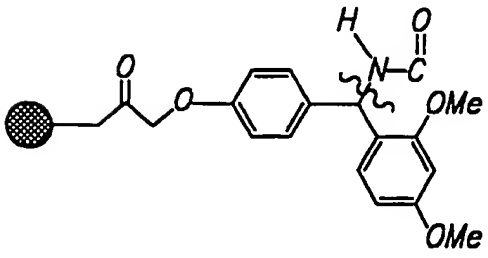
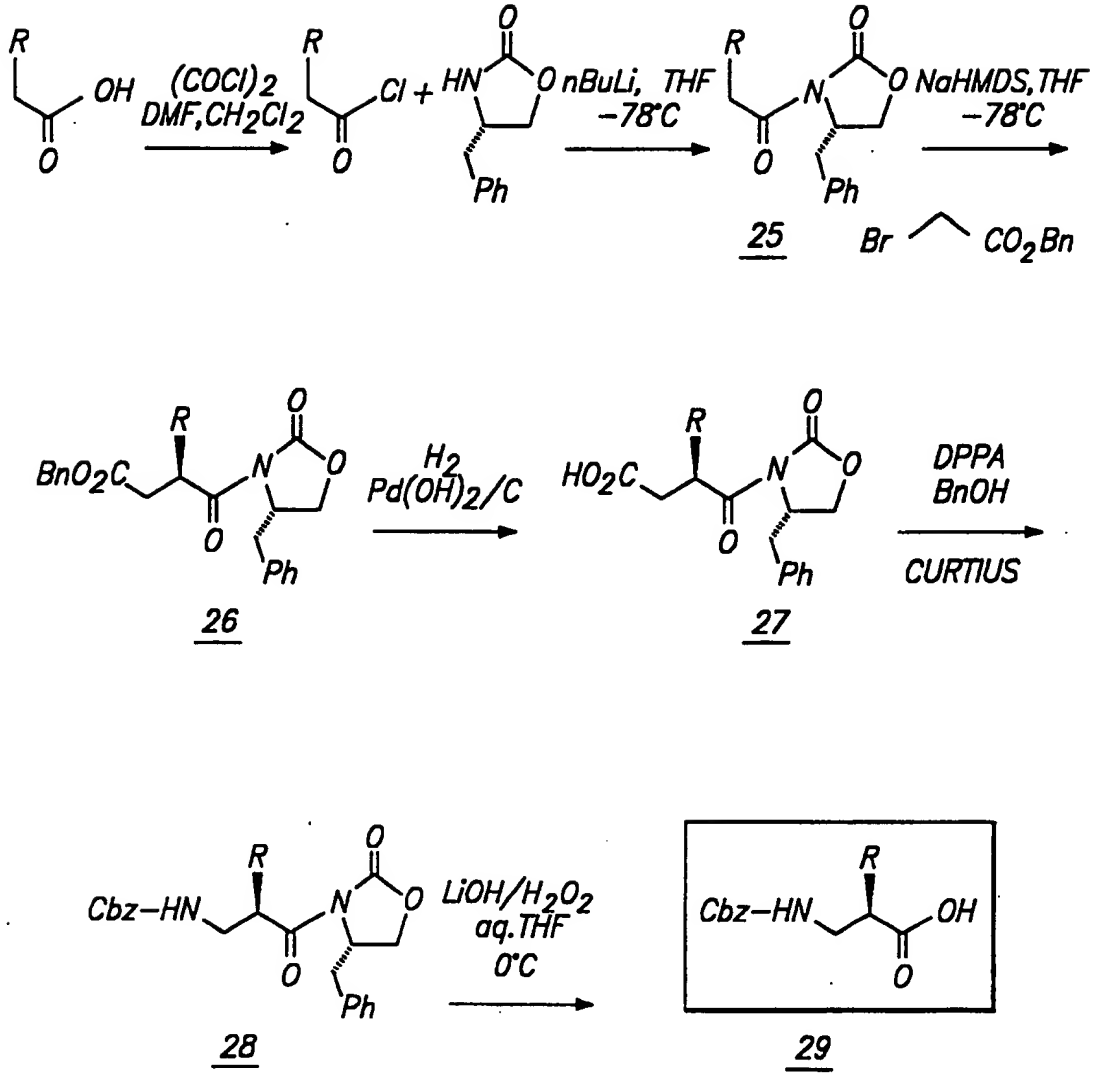


FIG. 1D

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Ph
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R
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O
O
Ph
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NaHMDS,THF
−78°C
Br
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BnO2C
R
N
O
O
O
Ph
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H2
Pd(OH)2/C
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N
O
O
Ph
27
DPPA
BnOH
CURTIUS
Cbz−HN
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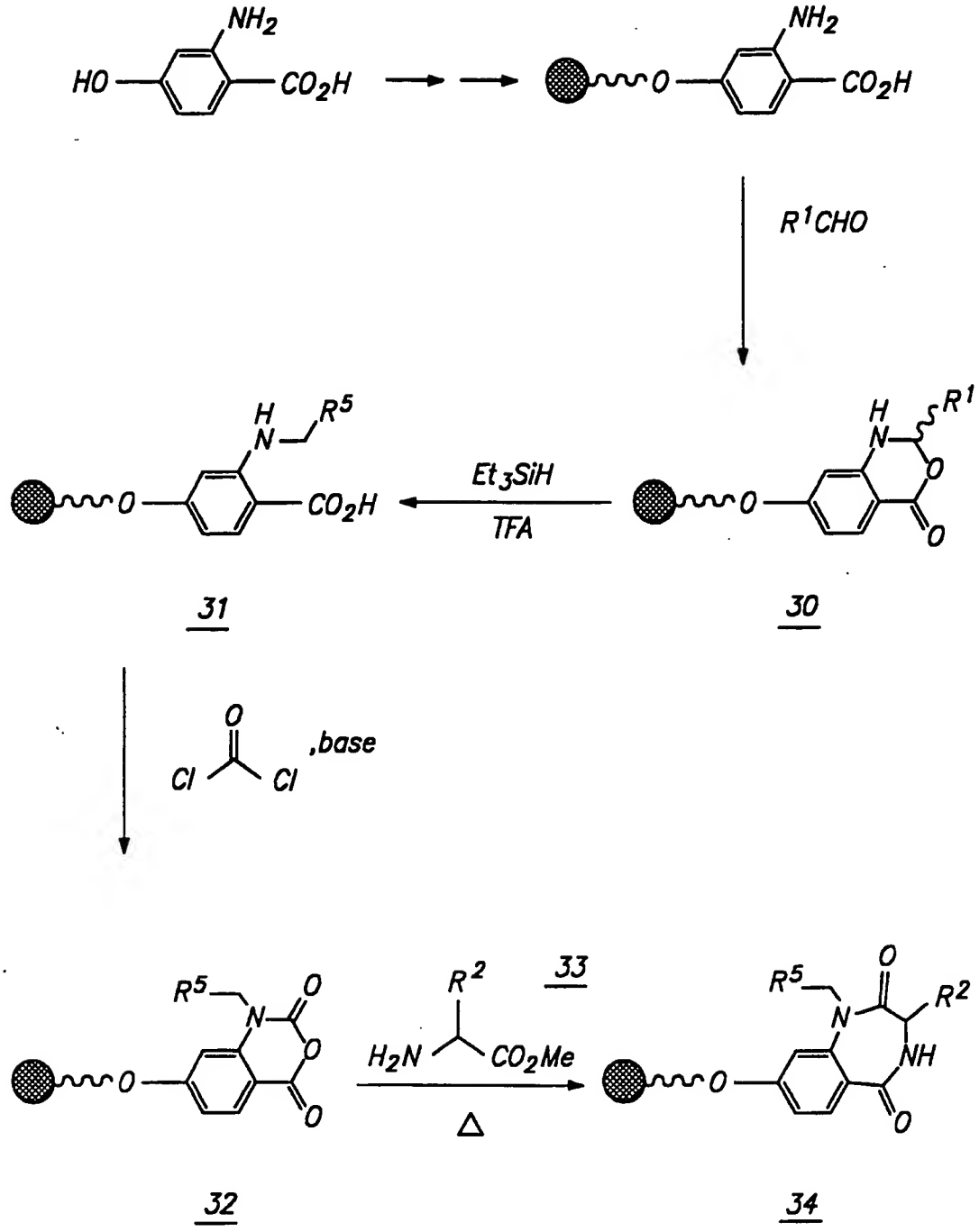
O
29

FIG. 2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R1
S
O
O
31
30
O
Cl
Cl
,base
R5
N
O
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R2
33
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CO2Me
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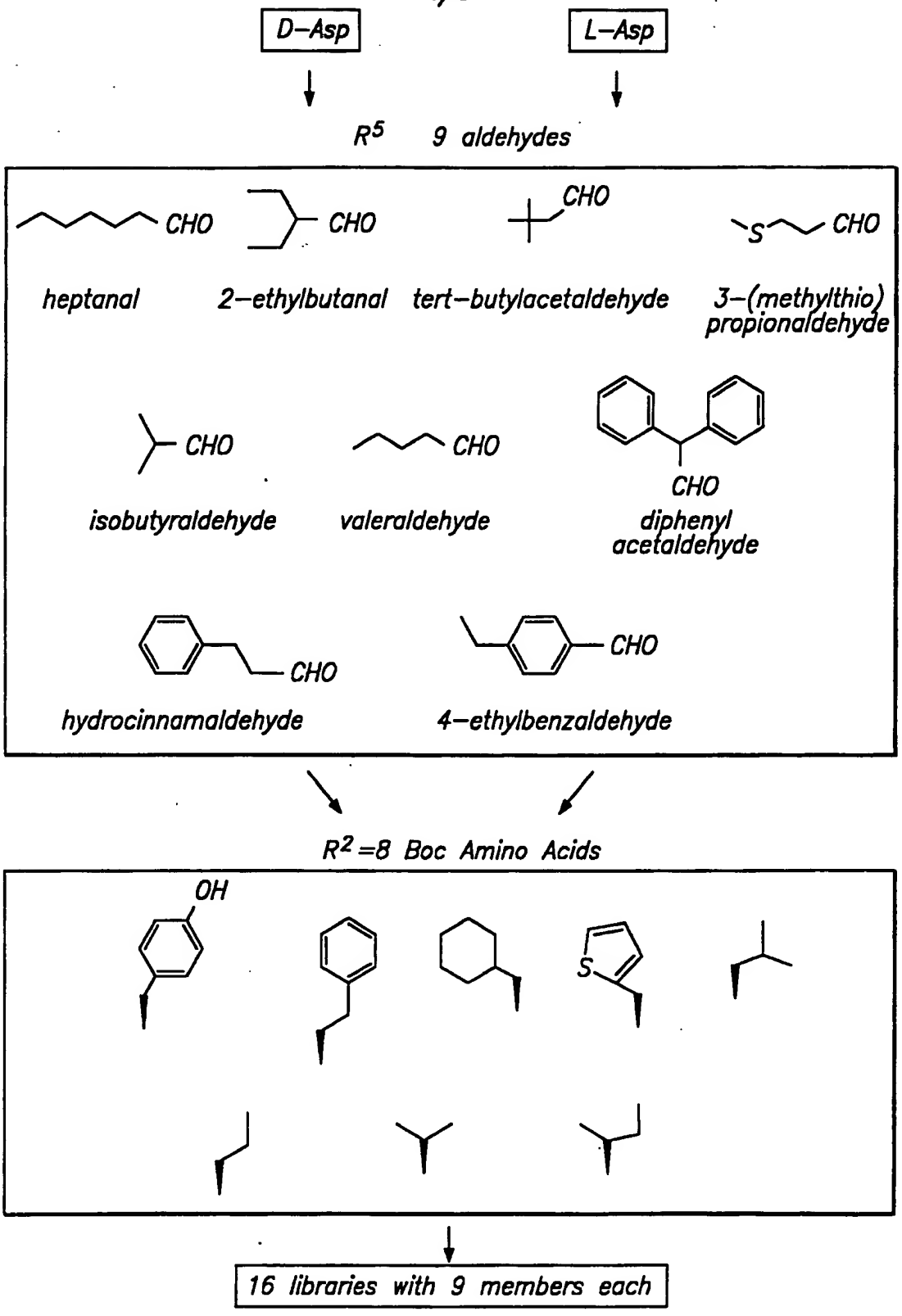
R2
NH
O
32
34

FIG. 3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L-Asp
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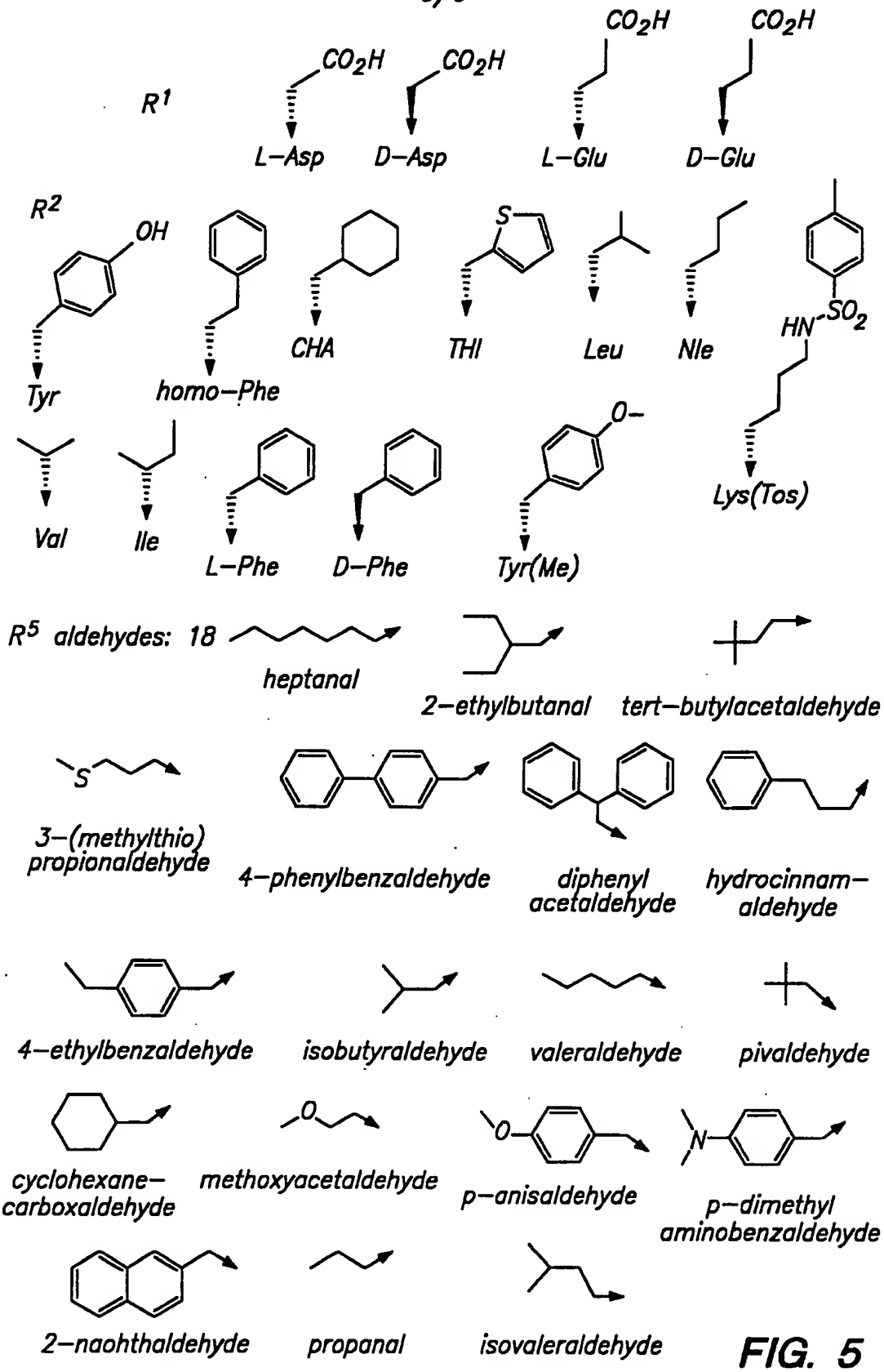
3-(methylthio) propionaldehyde
isobutyraldehyde
valeraldehyde
diphenyl acetaldehyde
hydrocinnamaldehyde
4-ethylbenzaldehyde
R^2=8 Boc Amino Acids
OH
S
16 libraries with 9 members each

FIG. 4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L–Glu
D–Glu
R2
OH
S
HN
SO2
Tyr
homo–Phe
CHA
THI
Leu
Nle
Lys(Tos)
Val
Ile
L–Phe
D–Phe
O–
Tyr(Me)
R5 aldehydes: 18
heptanal
2–ethylbutanal
tert–butylacetaldehyde
S
3–(methylthio) propionaldehyde
4–phenylbenzaldehyde
diphenyl acetaldehyde
hydrocinnam– aldehyde
4–ethylbenzaldehyde
isobutyraldehyde
valeraldehyde
pivaldehyde
cyclohexane– carboxaldehyde
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methoxyacetaldehyde
O
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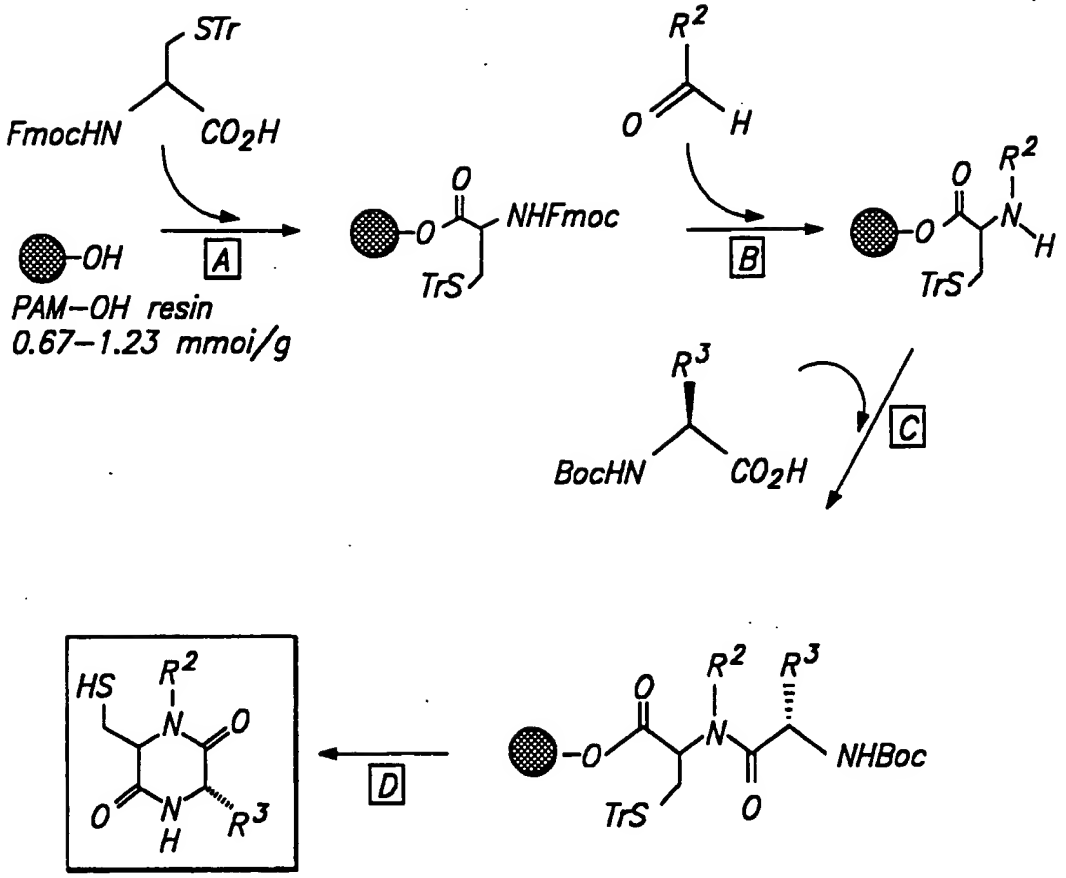
N
p–dimethyl aminobenzaldehyde
2–naohthaldehyde
propanal
isovaleraldehyde

FIG. 5



37

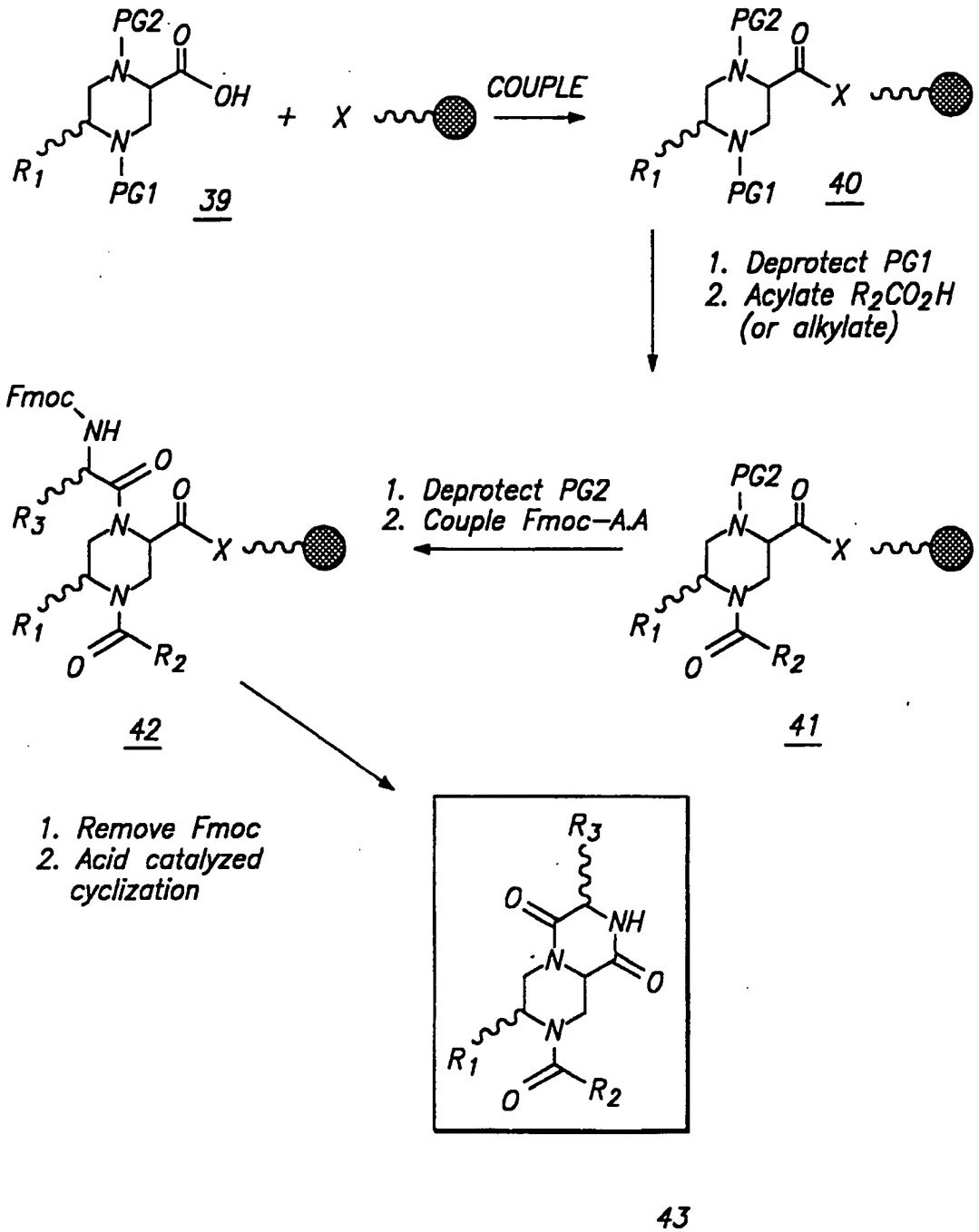
A. FmocAA, DIC, DMAP, DMF, 5h

B. 30% piperidine
–aldehyde, $NaCNBH_3$, $HC(OMe)_3$,
1% HOAc or MeOH

C. BocAA, HATU, DIEA, DCM/DMF, 2x12h

D. 95% TFA/TES
1% HOAc IN toluene 18h

FIG. 6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OH
+
X
COUPLE
R1
PG1
39
40
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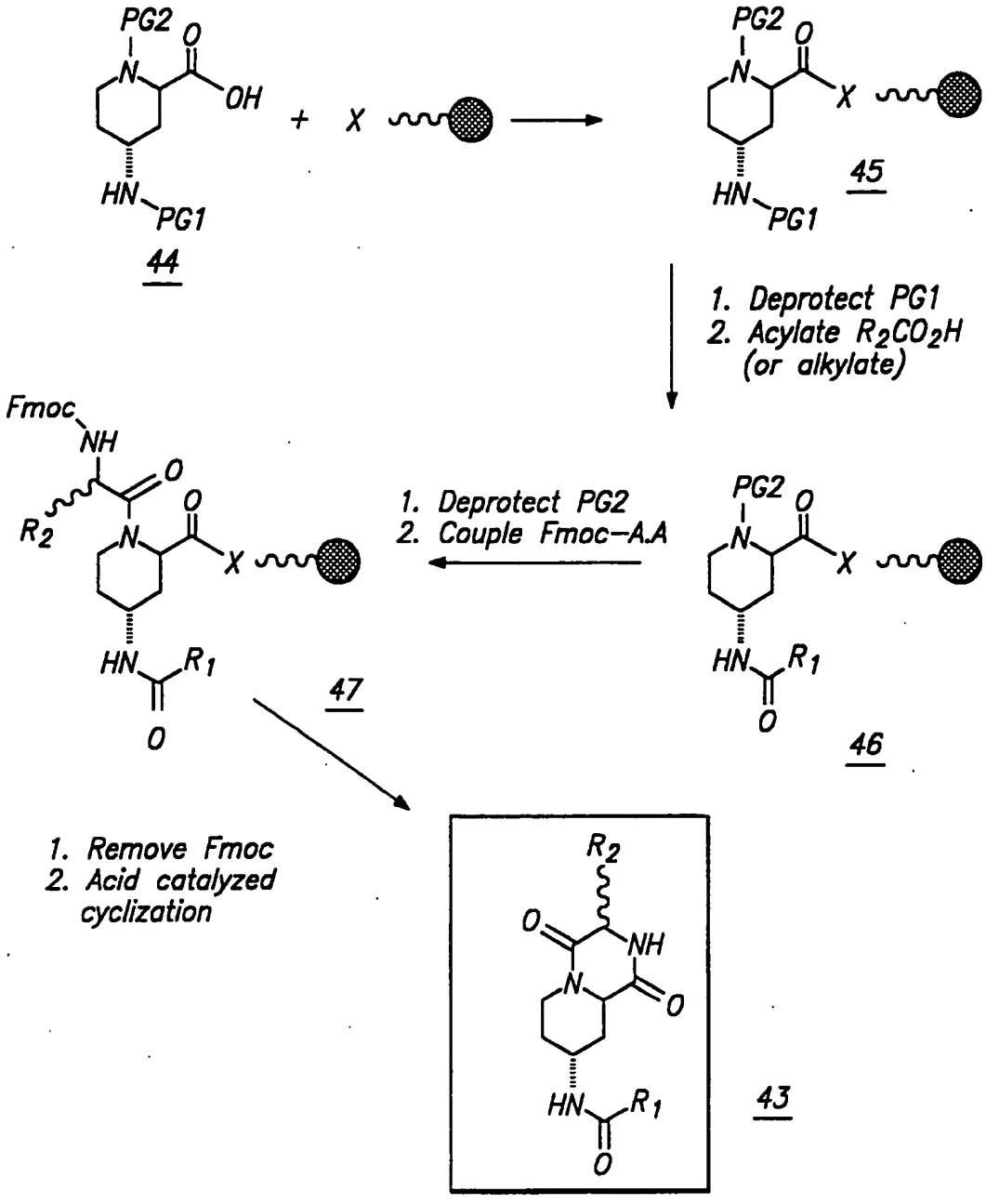
2. Acylate R2CO2H
(or alkylate)
Fmoc
NH
R3
1. Deprotect PG2
2. Couple Fmoc–A.A
R2
42
41
1. Remove Fmoc
2. Acid catalyzed
cyclization
43

FIG. 7


PG2
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N
OH
+
X
HN
PG1
44
45
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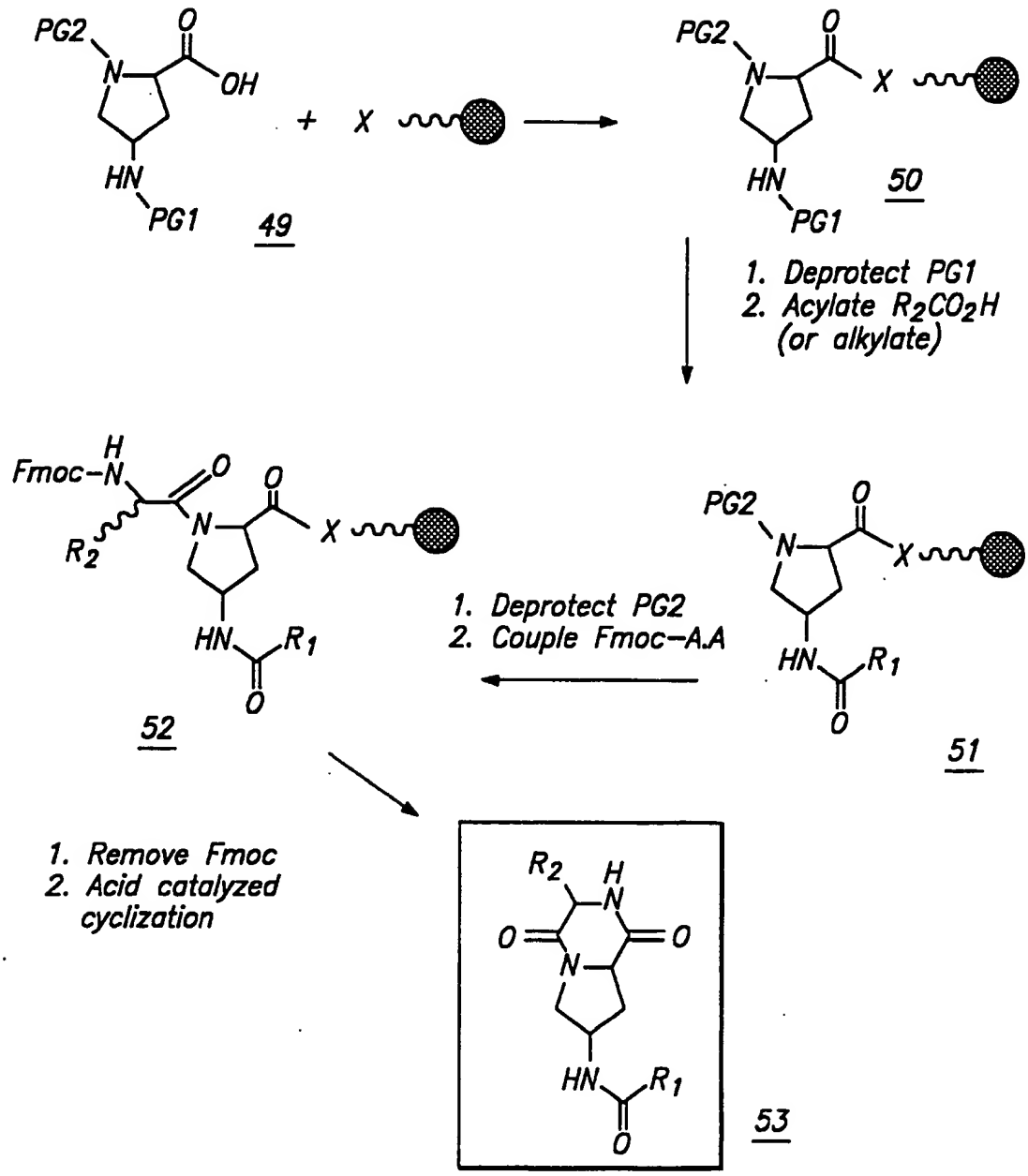
2. Acylate R_2CO_2H
(or alkylate)
Fmoc
NH
R_2
1. Deprotect PG2
2. Couple Fmoc–A.A
R_1
47
46
1. Remove Fmoc
2. Acid catalyzed
cyclization
43

FIG. 8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O
N
OH
HN
PG1
49
+ X
PG2
O
N
X
HN
PG1
50
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2. Acylate R_2CO_2H
(or alkylate)
Fmoc–N
H
O
O
N
X
R_2
HN
R_1
O
52
1. Deprotect PG2
2. Couple Fmoc–A.A
PG2
O
N
X
HN
R_1
O
51
1. Remove Fmoc
2. Acid catalyzed
cyclization
R_2
H
N
O
O
N
HN
R_1
O
53

FIG. 9

INTERNATIONAL SEARCH REPORT

In. ..ational application No.
PCT/US95/07964

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :G01N 33/53; 33/545; C07K 17/08; C07D 241/02
US CL :435/7.1; 436/518; 530/334; 544/358, 386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.1; 436/518; 530/334; 544/358, 386

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, STN
search terms: library, combinatorial, diketopiperazine, n-alkylated

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Tetrahedron: Asymmetry, volume 3, number 11, issued 1992, Kim et al. "Polymer attached cyclic dopeptides as catalysts for enantioselective cyanohydrin formation", pages 1421-1430, see schemes 1 and 2.	7-9 ----- 1-6, 10, 11, 15, 16, 29-31
Y --- A	Bioorganic & Medicinal Chemistry Letters, volume 3, number 5, issued 1993, Horwell et al., " The design of a dipeptide library for screening at peptide receptor sites", pages 799-802, see abstract.	1-6, 10, 11, 15, 16, 29-31 ---------- 12-14, 17-28
Y --- A	WO, A, 92/00091 (LAM ET AL.) 09 January 1992, see pages 20, 27, 30-32.	1-6, 10, 11, 15, 16, 29-31 ---------- 12-14, 17-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search
30 JULY 1995

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US95/07964

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, volume 89, issued June 1992, Brenner et al., "Encoded combinatorial chemistry", pages 5381-5383, see page 5383, column 1.	3
A	US, A, 5,324,483 (CODY ET AL.) 28 JUNE 1994, SEE EXAMPLE 8, SCHEME 8 AND TABLE 8.	1-31